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(54) Title: ASSAYS AND THERAPEUTIC METHODS BASED ON LYMPHOCYTE CHEMOATTRACTANTS

(57) Abstract

The present invention relates to a novel assay for lymphocyte chemotaxis. The assay is transendothelial assay using endothelial cells cultured on microporous filters. Lymphocyte transmigration through the filter toward a suspected chemoattractant is measured. Apparatuses for carrying out the assay are also provided. The apparatuses and methods of the present invention can be used for the identification of inhibitors (e.g., antagonists) or promoters (chemoattractants) of the adhesion receptor-mediated migration of leukocytes through the endothelium (extravasation). Such inhibitors and promoters respectively inhibit and promote the inflammatory response, and thus have therapeutic utilities. The inhibitors and promoters are identified by detecting their abilities to respectively inhibit or promote the chemotaxis of lymphocytes in the assay of the invention. The assay of the invention also has diagnostic utilities for detecting a disease or disorder involving a defect in lymphocyte chemotaxis. In a specific embodiment, the invention provides a novel lymphocyte chemoattractant, termed LCA, that is a variant of MCP-1 with a novelly processed aminoterminus. Derivatives and analogs of LCA, and antibodies and antibody fragments thereto are also provided. The invention also relates to therapeutic uses and compositions related to the foregoing.

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ASSAYS AND THERAPEUTIC METHODS BASED ON LYMPHOCYTE CHEMOATTRACTANTS

This application is a continuation-in-part of copending application Serial No. 08/030,764 filed March 12, 1993, which is incorporated by reference herein in its entirety.

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1. INTRODUCTION

The present invention relates to a novel assay for lymphocyte chemotaxis. The assay of the invention provides methods for screening for promoters (*e.g.*, chemoattractants) and inhibitors (*e.g.*, antagonists of chemoattractants) of lymphocyte chemotaxis. A novel lymphocyte chemoattractant, antibodies thereto, and therapeutic methods and compositions are also provided.

2. BACKGROUND OF THE INVENTION

Migration of leukocytes from blood vessels into diseased tissues is crucial to the initiation of normal disease-fighting inflammatory responses. But this process, known as leukocyte recruitment, is also involved in the onset and progression of debilitating and life-threatening inflammatory and autoimmune diseases. The pathology of these diseases results from the attack of the body's immune system defenses on normal tissues. Thus, blocking leukocyte recruitment to target tissues in inflammatory and autoimmune disease would be a highly effective therapeutic intervention. The leukocyte cell classes that participate in cellular immune responses include lymphocytes, monocytes, neutrophils, eosinophils and mast cells. Lymphocytes are "master cells" that control the activity of most of these other cell types, particularly the monocytes. Lymphocytes are the leukocyte class that initiate, coordinate, and maintain the inflammatory response, and thus are the most important cells to block from entering inflammatory sites. Lymphocytes attract monocytes to the site, which

are responsible for much of the actual tissue damage that occurs in inflammatory disease. Infiltration of these cells is responsible for a wide range of chronic, autoimmune diseases, and also organ transplant rejection. These diseases include rheumatoid arthritis, psoriasis, contact dermatitis, inflammatory bowel disease, multiple sclerosis, atherosclerosis, sarcoidosis, idiopathic pulmonary fibrosis, dermatomyositis, hepatitis, diabetes, allograft rejection, and graft-versus-host disease.

This process by which leukocytes leave the bloodstream and accumulate at inflammatory sites, and initiate disease, is best understood for neutrophils and monocytes, but is likely to be similar in broad outline for lymphocytes. This process takes place in at least three distinct steps (Springer, T.A., 1990, Nature 346:425-33; Lawrence and Springer, 1991, Cell 65:859-73; Butcher, E.C., 1991, Cell 67:1033-36). It is mediated at a molecular level by chemoattractant receptors, by cell-surface proteins called adhesion molecules, and by the ligands that bind to these two classes of cell-surface receptor. The major types of adhesion molecules are known as "selectins", "integrins" and "immunoglobulin (Ig) family" receptors.

Each of the three steps is essential for the emigration of the leukocytes to target tissues. Blocking the steps has been shown to prevent a normal inflammatory response, and impedes abnormal responses of inflammatory and autoimmune diseases (Harlan et al., 1992, *In vivo models of leukocyte adherence to endothelium. In Adhesion: Its Role in Inflammatory Disease.*, J.M. Harlan and D. Y. Liu, (eds.), W.H. Freeman & Co., pp. 117-150). The steps of leukocyte adhesion and transendothelial migration can be summarized as follows:

Step 1. Primary adhesion. Leukocytes attach loosely to the blood vessel endothelium and "roll" slowly along the blood vessel wall, pushed by the flow of blood. Leukocyte-endothelium attachment is mediated by cell surface adhesion molecules called "selectins" which bind to carbohydrate-rich ligands ("glycoconjugates") on the leukocyte cell surface.

Step 2. Activation of leukocytes and migration to the target site. Chemoattractant receptors on the surface of the leukocytes bind chemoattractants

secreted by cells at the site of damage or infection. Receptor binding activates the immune defenses of the leukocytes, and activates the adhesiveness of the adhesion molecules that mediate Step 3.

Step 3. Attachment and transendothelial migration. The leukocytes bind very tightly to the endothelial wall of the blood vessel and move to the junction between endothelial cells, where they begin to squeeze between these cells to reach the target tissue. This tighter binding is mediated by binding to adhesion receptors called "integrins" on the leukocytes to complementary receptors of the "Ig family" on the endothelium. (The Ig family molecules are named for their similarity to antibody molecules (immunoglobulins)). Chemoattractant receptors are also involved at this stage, as the leukocytes migrate up a concentration gradient of the chemoattractant secreted by cells at the target site.

These three classes of receptor-ligand interactions are all required and appear to act in a highly cooperative and coordinated manner to mediate leukocyte adherence to the microvasculature, diapedesis, and subsequent leukocyte mediated injury to tissue in inflammatory disease.

LFA-1 and Mac-1 together with p150,95 comprise the leukocyte integrins, a subfamily of integrins that share a common β subunit (CD18) and have distinct αL, αM and αX (CD11a, b and c) α subunits (reviewed in Larson and Springer, 1990, Immunol. Rev. 114:181-217; Springer, 1990, Nature 346:425-433). They are required for leukocyte emigration as demonstrated by an absence of neutrophil extravasation (1) in patients with mutations in the common β subunit (leukocyte adhesion deficiency), and (2) after treatment of healthy neutrophils with a monoclonal antibody (mAb) to the common β subunit *in vivo* or *in vitro* (reviewed in Anderson and Springer, 1987, Ann. Rev. Med. 38:175-194; Larson and Springer, 1990, Immunol. Rev. 114:181-217).

The integrins LFA (lymphocyte function-associated antigen)-1 and Mac-1 on the neutrophil bind to the Ig family member ICAM (intercellular adhesion molecule)-1 on endothelium (Smith et al., 1988, J. Clin. Invest. 82:1746-1756; Smith et al., 1989, J. Clin. Invest. 83:2008-2017; Diamond et al.,

1990, J. Cell Biol. 111:3129-3139). LFA-1 and not Mac-1 binds to ICAM-2 (de Fougerolles et al., 1991, J. Exp. Med. 174:253-267; Diamond et al., 1990, J. Cell Biol. 111:3129-3139), an endothelial cell molecule that is more closely related to ICAM-1 than these molecules are to other Ig superfamily members (Staunton et al., 1989, Nature 339:61-64).

The integrin VLA-4, that contains the $\alpha 4$ (CD49d) subunit noncovalently associated with the $\beta 1$ (CD29) subunit, is expressed by lymphocytes, monocytes, and neural crest-derived cells, and can interact with vascular cell adhesion molecule-1 (VCAM-1) (Elices et al., 1990, Cell 60:577). Like ICAM-1 and ICAM-2, VCAM-1 is a member of the Ig superfamily (Osborn et al., 1989, Cell 59:1203).

Chemoattractants are soluble mediators which activate cell adhesion and motility and direct cell migration through formation of a chemical gradient. They are produced by bacteria and numerous cell types including stimulated endothelial and stromal cells, platelets, tumor cells, cultured cell lines, and leukocytes themselves. The cells responding to chemoattractants appear to express specific receptors on their surfaces which bind the chemoattractant molecules and sense the gradient. Receptor stimulation induces cells to respond via a common signal transduction pathway which involves interaction of the chemoattractant-receptor complex with a guanine nucleotide or GTP-binding protein (G protein) (Gilman, A.G., 1987, Ann. Rev. Biochem. 56:615-49). This interaction stimulates phosphatidyl inositol hydrolysis by a phospholipase C, thus generating inositol phosphates and diacylglycerol. A transient rise in cytosolic free calcium then activates protein kinase C, and a variety of events including protein phosphorylation, membrane potential changes, and intracellular pH alterations ensue.

Several of the chemoattractants primarily affecting neutrophils were among the first chemoattractants identified. These include the complement component C5a, arachidonate derivative leukotriene B_4 (LTB_4), platelet activating factor (PAF), and formylmethionyl peptides of bacterial origin such as formyl-met-leu-phe (fMLP) (Devreotes and Zigmond, 1988, Annu. Rev. Cell Biol.

4:649-86). Although structurally dissimilar and stimulatory via separate receptors, these molecules produce a rapid and marked increase in neutrophil adhesiveness and motility leading to chemotaxis and prominent neutrophil accumulation *in vivo* (Pober and Cotran, 1990, Transplantation 50:537-44). The receptors for C5a and fMLP have been identified and sequenced; cDNA clones for each have also been generated (Gerard and Gerard, 1991, Nature 349:614-617; Boulay et al., 1990, Biophys. Res. Commun. 168:1103-09). These receptors share many structural features with one another and members of the "rhodopsin superfamily" of protein receptors (Dohlman et al., 1991, Ann. Rev. Biochem. 60:653-88).

More recently, a protein chemoattractant for neutrophils designated neutrophil activating protein-1 (NAP-1) or interleukin 8 (IL-8) was identified and molecularly cloned (Yoshimura et al., 1987, Proc. Natl. Acad. Sci. USA 84:9233-37; Matsushima et al., 1988, J. Exp. Med. 167:1883-93; Oppenheim et al., 1991, Annu. Rev. Immunol. 9:617-48). IL-8 was originally characterized as a 72 amino acid molecule produced by monocytes; variants of 79, 77, and 69 amino acids have subsequently been identified from additional sources including activated endothelial cells, lymphocytes, fibroblasts, and tumor lines. IL-8 has structural homology to a supergene family of novel 8-10 kDa cytokines isolated chiefly by subtractive hybridization (Oppenheim et al., 1991, Annu. Rev. Immunol. 9:617-48) and recently named the "chemokine" family. IL-8 and several other human cytokines, including platelet factor 4, platelet basic protein, IP-10, and melanoma growth stimulating factor/GRO comprise a subfamily of chemokines located on chromosome 4. In this subfamily, the relative positions of four cysteine residues are identical, with the first two cysteine residues separated by a single amino acid (C-X-C). Disulfide bonds between these four cysteines form two loops which appear to be essential for activity. The other subfamily, which includes the monocyte chemoattractants RANTES and monocyte chemoattractant protein-1 (see below), is clustered on chromosome 17, and the first two cysteines are adjacent (C-C).

The biological profile of activity for IL-8 is similar to that for C5a, LTB_4, PAF, and fMLP; the respiratory burst is induced, neutrophil chemotaxis is promoted, and Mac-1 expression is increased on the surface of granulocytes (Baggiolini et al., 1989, J. Clin. Invest. 84:1045-49; Detmers et al., 1990, J. Exp. Med. 171:1155-62). IL-8 differs from these others, however, in that it has been reported to attract approximately 10% of human peripheral blood T lymphocytes of either $CD4^+$ or $CD8^+$ subsets (Leonard et al., 1990, J. Immunol. 144:1323-30; Larsen et al., 1989, Science 241:1464-66), but does not attract monocytes. There is some controversy on whether IL-8 is a lymphocyte chemoattractant, because when injected in human skin it attracts neutrophils but not lymphocytes (Leonard et al, 1991, J. Invest. Dermatol. 96:690-94).

The chemoattractants which predominantly activate and guide monocytes include monocyte chemoattractant protein-1 (MCP-1) (Leonard and Yoshimura, 1990, Immunol. Today 11:97-101), the RANTES protein (Schall et al., 1990, Nature 347:669-71), and the neutrophil α granule protein CAP37 (Pereira et al., 1990, J. Clin. Invest. 85:1468-76), among others. As noted above, MCP-1 and RANTES are structurally homologous and belong to the subfamily of chemoattractive cytokines that are defined by a configuration of four cysteine residues in which the first two are adjacent (C-C). CAP37's structure is most homologous to proteins of the serine protease family (Pereira et al., 1990, J. Clin. Invest. 85:1468-76). The 76 amino acid MCP-1 is produced by activated endothelium, lymphocytes, macrophages, fibroblasts, smooth muscle cells, and tumor cells. It binds only to monocytes and induces approximately 30% of peripheral blood monocytes to respond in chemotaxis assays. The 68 amino acid RANTES is of special interest because it has been reported to selectively attract memory T helper cells ($CD4^+$ and UCHL1 antigen/CD45RO positive) as well as monocytes. Of the chemoattractants known to attract lymphocytes, only RANTES appears subset-selective. However, the lymphocyte chemoattractive activity of RANTES may be weaker than its activity for eosinophils (Kameyoshi et al., 1992, J. Exp. Med. 176:587-92).

Endothelial cells cultured on matrices of type I collagen have been used to study neutrophil migration toward the chemoattractant IL-8 (Huber et al., 1991, Science 254:99-102). When tested with IL-8 contained in conditioned media from stimulated endothelial cells, neutrophils migrated similarly, and there was a similar signal-to-noise ratio, on collagen matrix plus endothelium and on matrix alone. Thus, there was no indication that the assay would be improved for other cell types by adding endothelium. Indeed, the expectation would have been that neutrophils and lymphocytes would behave similarly. Lymphocyte migration across endothelium into collagen gels has previously been reported in assays of migration (Kavanaugh et al., 1991, J. Immunol. 146:4149-4156; Masuyama et al., 1992, J. Immunol. 148:1367-1374). In this system, the endothelium is cultured on a collagen gel formed on a culture dish or well for several days, then lymphocytes are added. This is an assay of migration in which there is no evidence that chemotaxis is involved; rather, it appears to assay for a migratory subset of lymphocytes. There is no teaching of a chemoattractant. Furthermore, lymphocytes migrate into collagen gels even in the absence of an endothelial cell monolayer.

Compared with neutrophil and monocyte chemoattractants, little is known about chemoattractants for lymphocytes. The best characterized lymphocyte chemoattractants are RANTES and IL-8, which primarily attract monocytes and neutrophils, as noted above. Several *in vitro* studies have described lymphocyte chemotactic activities in the culture supernatants of mixed lymphocyte reactions and mitogen-stimulated human peripheral blood mononuclear cells (Cruikshank and Center, 1982, J. Immunol. 128:2569; Center and Cruikshank, 1982, J. Immunol. 128:2563-68; Van Epps et al., 1983, J. Immunol. 130:2727; Van Epps et al., 1983, J. Immunol. 131:687). One of these activities was related to a protein of apparent molecular weight 14 kD named lymphocyte chemotactic factor (LCF) (Cruikshank and Center, 1982, J. Immunol. 128:2569; Center and Cruikshank, 1982, J. Immunol. 128:2563-68) that was produced by T cells (Van Epps et al., 1983, J. Immunol. 130:2727). The initial purifications of LCF possibly yielded activities contaminated by the chemotactic

mediators interleukin-1 and -2 (IL-1 and IL-2), however (Cruikshank and Center, 1982, J. Immunol. 128:2569). Subsequent purifications using high performance liquid chromatography have separated IL-1 and IL-2 from a LCF 10.5 kD in size (Potter and Van Epps, 1987, Cell. Immunol. 105:9-22). This LCF attracts lymphocytes, but selective subset chemotaxis was not assessed. Further characterization of the 10.5 kD LCF, including sequence information, has not been published. Another lymphocyte chemoattractant factor has been described that is 56,000 M_r, binds to CD4, and is selectively chemoattractive for the CD4 subset of lymphocytes (Cruikshank et al., 1991, J. Immunol. 146:2928-34). It also is chemoattractive for monocytes (Cruikshank et al., 1991, J. Immunol. 146:2928-34) and eosinophils (Rand et al., 1991, J. Exp. Med. 173:1521-28). More recently, a less than 1 kD molecule that was extractable in lipid solvents (*i.e.*, not a peptide) was isolated from normal human skin and named plasma-associated lymphocyte chemoattractant (Bacon et al., 1990, Eur. J. Immunol. 20:565-71). It has been suggested that this molecule is constitutively expressed in skin and accounts for surveillance lymphocyte trafficking there (Bacon et al., 1990, Eur. J. Immunol. 20:565-71). A variety of other agents or poorly characterized activities have also been reported. These include fetal calf serum, the protein casein, the mitogen phytohemagglutinin, and supernatants of stimulated peritoneal macrophages (Berman et al., 1988, Immunol. Invest. 17:625-77).

Although considerable effort has been invested on the study of lymphocyte chemoattractants, they remain poorly characterized relative to monocyte and neutrophil chemoattractants. Chemoattractants for the latter cell types, such as MCP-1 and IL-8, have been purified based on the conventional chemotaxis assay, sequenced, and cloned. However, no molecule identified primarily as a lymphocyte chemoattractive factor has been sequenced and cloned.

In large measure, the lack of rapid progress on lymphocyte chemoattractants appears due to the unreliability or lack of biological relevance of currently available lymphocyte chemotaxis assays. For example, the protein casein, fetal calf serum, the mitogen phytohemagglutinin, and the hormone

insulin, have all been reported active in lymphocyte chemotaxis assays (Berman et al., 1988, Immunol. Invest. 17:625-77), but the first three are not physiologic chemoattractants, and it seems doubtful that insulin is, because lymphocytes do not accumulate at sites of insulin injection in diabetics. Currently available lymphocyte chemotaxis assays have been reviewed (Berman et al., 1988, Immunol. Invest. 17:625-77). The most widely used is the Boyden Chamber assay, in which a microporous membrane divides two chambers, the lower containing the test chemoattractant and the upper containing the cells, *e.g.* lymphocytes. The microporous membrane is commonly nitrocellulose or polycarbonate, and may be coated with a protein such as collagen. The distance of migration into the filter, the number of cells crossing the filter that remain adherent to the undersurface, or the number of cells that accumulate in the lower chamber may be counted.

3. SUMMARY OF THE INVENTION

The present invention relates to a novel assay for lymphocyte chemotaxis. The assay is a transendothelial assay using endothelial cells cultured on microporous filters. Lymphocyte transmigration through the filter toward a known or suspected chemoattractant is measured. Apparatuses for carrying out the assay are also provided.

The apparatuses and methods of the present invention can be used for the identification of inhibitors (*e.g.*, antagonists) or promoters (chemoattractants) of the adhesion receptor-mediated migration of leukocytes through the endothelium (extravasation). Such inhibitors and promoters respectively inhibit and promote the inflammatory response, and thus have therapeutic utilities. The inhibitors and promoters are identified by detecting their abilities to respectively inhibit or promote the chemotaxis of lymphocytes in the assay of the invention. The assay of the invention also has diagnostic utilities for detecting a disease or disorder involving a defect in lymphocyte chemotaxis.

In a specific embodiment, the invention provides a novel lymphocyte chemoattractant, termed LCA, that is variant of MCP-1 having a

novelly processed amino-terminus. Derivatives and analogs of LCA, and antibodies and antibody fragments thereto are also provided. The invention also relates to therapeutic uses and compositions related to the foregoing.

3.1. DEFINITIONS

As used herein, the following terms shall have the indicated meanings:

EC	=	endothelial cells
FITC	=	fluorescein isothiocyanate
fMLP	=	formyl-Met-Leu-Phe
HUVEC	=	human umbilical vein endothelial cells
IL-8	=	interleukin 8
mAb	=	monoclonal antibody
MLR	=	mixed lymphocyte reaction
PBL	=	peripheral blood lymphocytes
PBMC	=	peripheral blood mononuclear cells
PHA	=	phytohemagglutinin

4. DESCRIPTION OF THE FIGURES

Figure 1. A conventional (A) and a transendothelial (B) chemotaxis assay. (A) Transwell® with 8 μm polycarbonate membrane plus or minus ICAM-1, fibronectin, or collagen on the filter. The conventional assay does not detect specific migration of lymphocytes, while monocytes and neutrophils migrate specifically. (B) Transwell® with endothelial cell monolayer grown on collagen-coated 8 μm filter. A transendothelial assay of the invention detects specific migration of lymphocytes; a high ratio of migration to chemoattractant is observed relative to control media (often greater than a tenfold difference). Exemplary protocol for transendothelial chemotaxis assay: (1) Grow endothelial cell monolayer on collagen-coated, 8 μm polycarbonate Transwell® filter. (2) Add lymphocytes to upper chamber, chemoattractant to bottom chamber. (3) Remove cells from bottom chamber 1 to 4 hours later and count.

Alternatively, or in addition, lymphocytes may be labeled, *e.g.*, by a fluorescent label, such that migrating cells may be quantitated by fluorescence detection methods.

Figure 2. Schematic diagram of an exemplary apparatus for the transendothelial chemotaxis assay of the invention.

Figure 3. Neutrophil and monocyte chemotaxis through uncoated Transwell® filters. Using the conventional chemotaxis assay, neutrophil migration to known neutrophil chemoattractants and monocyte migration to known monocyte chemoattractants was measured. A) Neutrophil chemotaxis was through 3 μm pore Transwells®. B) Monocyte chemotaxis was through 5 μm pore Transwells®. Interleukin 8 (IL-8), RANTES (both from Pepro-Tech, Rocky Hills, NJ) and formyl-Met-Leu-Phe (fMLP) (Sigma) were used at indicated concentrations. All dilutions were in assay media which in these experiments was L-15 (Gibco) plus 1% HSA. Data are presented as average number of cells migrating per grid. Five grids per sample were counted and all samples were assayed in duplicate.

Figure 4. Lymphocyte migration through uncoated (A) or endothelial cell-coated (B) Transwells®. A) Lymphocyte migration to mixed lymphocyte reaction (MLR) supernatant or media conditioned by phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) was assayed with uncoated Transwells® using the conventional chemotaxis assay. Percent of migrating cells was calculated by counting the number of cells in the bottom chamber of each sample in a hemocytometer. The original input number of cells was also counted by hemocytometer and served as the input standard. B) Lymphocyte migration to the same supernatants was assayed using endothelial-coated Transwells® in the transendothelial chemotaxis assay. Five 10 x 10 grids of fluorescently labeled cells were counted for each sample and for an input cell number control. Percent of input cells migrating into the bottom chamber is presented.

Figure 5. Endothelial cell monolayers act as a barrier to diffusion of fluorescein isothiocyanate (FITC)-dextran. FITC-dextran (Sigma) was diluted

1:500 in assay media and 600 μl was added to three wells of a 24-well tissue culture plate; 600 μl of assay media alone was added to a fourth well. A confluent endothelial cell-coated, 8 μm Transwell® insert was placed in one FITC-dextran well and a non-endothelial cell-coated, 8 μm Transwell® insert was placed in the second FITC-dextran well. One hundred microliters of assay media was added to the top of both Transwells® and to the third FITC-dextran well, which served as the equilibrium control well. The plate was incubated at 37°C, 5% CO_2. After 15 min, 50 μl of media was removed from the top chamber of each Transwell® insert and from the medium and equilibrium control wells. Each sample was diluted 1:1 with assay media in a 96-well polystyrene microtiter plate (Linbro-Titertek; Flow Laboratories, McLean, VA). Fluorescence was directly quantitated from the 96-well plate using a Pandex fluorescence concentration analyzer (Idexx Corp., Westbrook, ME) (de Fougerolles et al., 1991, J. Exp. Med. 174:253-267). Meanwhile, 50 μl of fresh assay media was added to the top chamber of each Transwell® and control well to replace the aliquot previously removed. Samples were analyzed again after 4 hr.

Figure 6. Dose response of lymphocyte transendothelial chemotaxis to MLR supernatant. Chemotactic potential of MLR supernatant was assessed using the transendothelial chemotaxis assay. MLR supernatant was diluted serially in assay media and assay media was used as the media control.

Figure 7. Migration of peripheral blood lymphocytes (PBL) to potential chemoattractants through endothelial-coated Transwells®. The transendothelial chemotaxis assay was used to measure the chemotactic potential of cytokines or supernatant from cytokine-stimulated endothelial cells. Supernatants were harvested after 20 hr from rh-IL-1α (10 U/ml; Genzyme Corp., Cambridge, MA) or rh-IL-4 (30 U/ml; Genzyme)-stimulated endothelial cells. Supernatants were used undiluted in the chemotaxis assay. Recombinant human cytokines and fMLP (Sigma) were diluted, as indicated, in assay media. Endothelial cell culture media and MLR control media was as described in the text. EC: endothelial cells. Sup: Supernatant.

Figure 8. $CD3^+$ T lymphocytes and $CD14^+$ monocytes among PBL migrating through endothelial-coated 8 μm Transwells®. The transendothelial chemotaxis assay was modified as follows. Endothelial cells were grown on 24.5 mm diameter, 8 μm Transwell® filters. Chemotactic factors were added to the bottom chambers in a final volume of 2 ml and 1 ml of PBL at 5 x 10^6 cells/ml was added to each top chamber. Chemotactic factors were diluted in assay media to the concentrations indicated (MCP-1 and RANTES, PeproTech, Inc., Rocky Hills, NJ; MIP-1α, R&D Systems, Minneapolis, MN). Following the 4 h incubation, the migrated cells in duplicate samples were harvested, washed, and resuspended in 30 μl of L-15/2% FCS. Ten microliters of each sample were added to 10 μl of monoclonal antibody (mAb) in a 96-well round bottom titertek plate. The mouse monoclonal antibodies used were X-63 (non-binding antibody control) culture supernatant, OKT3 (anti-CD3; used at 10 μg/ml), and My4 (anti-CD14; used at 10 μg/ml). After a 30 min incubation at 4°C, the cells were washed 3X and incubated with 10 μl of 1:10 diluted FITC-labeled goat anti-mouse IgG (H & L; Zymed Immunochemicals) for 30 min at 4°C on a plate shaker. The cells were washed 3X and fixed with 2% paraformaldehyde/PBS plus EPICS Immuno-Brite fluorescent beads (Coulter Diagnostics) at a concentration of 1.3 x 10^6 beads/ml. Samples were analyzed using an Epics V flow cytometer. The number of positive cells for each antibody was determined by reference to the number of Immuno-Brite beads in each sample. Solid bars: $CD3^+$ cells; open bars: $CD14^+$ cells.

Figure 9. $CD3^+$ lymphocytes, rather than $CD14^+$ monocytes, migrate towards MLR supernatant or supernatant from PHA-stimulated PBMC. The transendothelial chemotaxis assay was performed using the modifications described for Figure 7. Cells migrating to MLR supernatant or supernatant from PHA-stimulated PBMC were collected from the bottom of the wells and stained with anti-CD3 or anti-CD14 antibodies, as described for Figure 7. Solid bars: $CD3^+$ cells; open bars: $CD14^+$ cells.

Figure 10. Heparin-Sepharose Affinity Chromatography of PHA Supernatant. PHA-supernatant was applied to heparin-Sepharose and the column

washed and eluted with a NaCl gradient as described in Section 7.1. Fractions were tested in duplicate at a 1/40 dilution in the transendothelial chemotaxis assay. Active fractions were pooled as shown by the bar. The response to 30x-concentrated PHA-supernatant (1/100 dilution) and to assay media control is shown. The experiment shown is representative of four purifications. Percent lymphocyte migration is indicated by the filled diamonds, protein concentration by the unfilled squares (OD_{280}), and salt (NaCl) concentration by the dashed line.

Figure 11. Gel Filtration Chromatography on G-75 Sephadex. Pooled material from heparin-Sepharose chromatography was subjected to G-75 Sephadex gel filtration and fractions were assayed at 1/20 dilution in the transendothelial chemotaxis assay. The elution positions of molecular weight standards are shown by A, bovine serum albumin (66 kD); B, carbonic anhydrase (29 kD); and C, cytochrome c (12.4 kD). Active fractions were pooled as shown by the bar. The response to 30x-concentrated PHA-supernatant (1/100 dilution) and to assay media control is shown. The experiment shown is representative of four purifications. Chemotactic activity in the transendothelial chemotaxis assay is presented as percent of maximal migration (filled diamonds). Protein levels are shown as absorbance at 280 nm (open squares).

Figure 12. Reverse Phase HPLC. Material from the G-75 column was applied and eluted with three gradients of acetonitrile in 0.1% trifluroacetic acid (TFA)/H_2O. Only the first half of the chromatograph is shown as few significant protein peaks eluted in the second half. Fractions eluting between 25-35% acetonitrile were assayed for chemoattractant activity since previous reverse phase chromatography experiments had localized all of the chemotactic activity to this region (data not shown). One tenth of each fraction was lyophilized and one half of each lyophilized fraction was used per duplicate in the transendothelial chemotaxis assay. The experiment shown is representative of three purifications. Percent lymphocyte migration in the transendothelial chemotaxis assay is indicated by the hatched bars; absorbance at 214 nm is indicated by the solid line, and the acetonitrile gradient is indicated by the dashed line.

Figure 13. Transendothelial Chemotaxis of PBL and Purified T Lymphocytes. Transendothelial chemotaxis of PBL (panel A) and T lymphocytes (panel B) was assayed in response to media controls, recombinant human MCP-1 (20 ng/ml), fMLP (10^{-8} M), and 30x-concentrated PHA-supernatant (1/100 dilution). The experiment shown is representative of three experiments. Error bars show the range of duplicates.

Figure 14. T Lymphocytes Respond to Recombinant MCP-1 in a Dose-Dependent Manner. Transendothelial migration of purified T lymphocytes in response to increasing doses of recombinant MCP-1 was assessed. MCP-1 was assayed at the concentrations indicated; fMLP was 10^{-8} M. The experiment shown is representative of three experiments. Bars show the range of duplicates.

Figure 15. Antibody to MCP-1 is Able to Neutralize Lymphocyte Chemotactic Activity in PHA Supernatant. Transendothelial migration of purified T lymphocytes to recombinant MCP-1 or PHA-supernatant was measured in the presence or absence of neutralizing rabbit anti-human MCP-1 polyclonal antibody or normal rabbit IgG control. MCP-1 was assayed at a concentration of 20 ng/ml; PHA-supernatant was assayed at a 1/10 dilution; fMLP was assayed at 10^{-8} M. This experiment is representative of three experiments. Bars show the range of duplicates.

Figure 16. Ability of PBL or T Lymphocytes to Migrate to MCP-1 Through Transwells With or Without Endothelial Monolayers. The ability of cells to migrate to recombinant MCP-1 or PHA-supernatant (1/30 dilution) through Transwells without (panel A) or with endothelial monolayers (panel B) was assessed. fMLP was used at 10^{-8} M. This experiment is representative of four experiments. Bars show the range of duplicates.

Figure 17. Phenotype of Lymphocytes Migrating to MCP-1 Through Endothelial Monolayers. Starting input cells (thin line) and cells migrating to MCP-1 (thick line; 50 ng/ml) were collected at 4 hr, reacted with antibodies, and subjected to three-color flow cytometry as described in Section 7.1. PE-coupled antibodies to monocyte and endothelial cell antigens were used to determine that the scatter-gated population included only lymphocytes. The

CD14 panel is included to confirm that the gated populations of cells are free of monocytes. Histograms are from one of two similar experiments using three-color immunofluorescence. Similar results were obtained in six experiments using single-color indirect immunofluorescence.

Figure 18. Amino acid sequence of MCP-1 and of LCA. Amino acid numbers 1-76 depict the known amino acid sequence of MCP-1 (Yoshimura and Leonard, 1992, in *Interleukin 8 (NAP-1) and Related Chemotactic Cytokines*, Cytokines, Vol. 4, Baggiolini and Sorg (eds.), Basel, Karger, pp. 131-152). The amino acid sequence of LCA, the variant of MCP-1 with a novelly processed amino-terminus, is shown in the figure from amino acid numbers 3-76. LCA was purified from mitogen-stimulated PBMC and subjected to amino acid sequencing as described in Section 7.1.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1. A TRANSENDOTHELIAL LYMPHOCYTE CHEMOTAXIS ASSAY

We have developed a novel assay for lymphocyte chemotaxis. The assay is a transendothelial assay that allows quantitation of cells accumulating in the chamber containing the chemoattractant, has a high signal-to-noise ratio, is highly reliable, and provides a method of screening for and purification of novel chemoattractants (Fig. 1). The assay has utility for identification and monitoring of purification of chemoattractants, and in screening for antagonists of lymphocyte chemoattraction (chemotaxis inhibitors).

The invention is based in part on our discovery that lymphocytes behave differently than monocytes and neutrophils in chemotaxis chambers. Neutrophils migrate vigorously to the formylpeptide f-Met-Leu-Phe and IL-8, and monocytes respond well to f-Met-Leu-Phe and much less so to RANTES. The response is readily measurable by counting the number of cells that appear in the bottom chamber. By contrast, only a small percentage of lymphocytes migrate through polycarbonate filters with 5 μm pores, whereas a high percentage of cells (30-40%) migrate or fall through 8 μm pores, whether or not chemoattractant is added. The same 8 μm filters in the same geometry are used by other

investigators to assay for chemotaxis by counting the number of cells that migrate through the filter and stick to the underside (Schall et al., 1990, Nature 347:669-671). However, our results show that these must be numerically a very small percentage of the cells that fall or migrate through the filter. Therefore, these may be assays of sticking rather than chemotaxis.

We have developed an excellent lymphocyte chemotaxis assay by culturing endothelium on microporous filters coated with collagen or fibronectin, and assaying lymphocyte transmigration toward chemoattractants.

The results with the transendothelial chemotaxis assays of the invention are in sharp contrast to assays with microporous membranes alone (see Section 6). The transendothelial chemotaxis assay system accurately recapitulates lymphocyte emigration from blood vessels toward chemoattractants *in vivo*, in which cells migrate through endothelium and basement membrane into tissues. We thus term these assay chambers a type of "artificial vessel construct."

Chemokinesis is defined as stimulated motility that is random in direction, whereas chemotaxis is directional towards the stimulus. The activity we have defined is directional, because "checkerboard assays" (see Zigmond and Hirsch, 1973, J. Exp. Med. 137:387-410) show that lymphocytes migrate when chemoattractant is present in the bottom chamber and not in the top, but that migration falls off as chemoattractant is added to the top chamber (see Section 6).

The artificial vessel construct for use in the lymphocyte chemotaxis assay of the invention comprises a filter on which an endothelial cell monolayer consisting of living (*e.g.*, not fixed) cells is situated. The filter is a microporous filter, of pore size in the range of about 3-8 microns, preferably 5-8 microns. In specific embodiments, the filter pore size is in the range of 4-7 microns, or is 5 or 8 microns. The filter is polycarbonate, nitrocellulose, or other polymer, and is preferably polycarbonate.

To produce an artificial vessel construct of the invention, a composition such as one comprising fibronectin or collagen is placed on the filter so as to coat the filter's surface, in order to allow attachment of endothelial cells to the filter. In a preferred embodiment, type I collagen is applied to the filter.

Fibronectin and collagen can be obtained and/or purified by use of any methods known in the art. Compositions comprising fibronectin or collagen are also commercially available. For example, fibronectin can be purchased from Telios, Inc. (La Jolla, CA); type I collagen can be purchased from Organogenesis, Inc. (Canton, MA).

The filter coated with the fibronectin or collagen composition is preferably then incubated for a short time after coating (*e.g.*, at least about one-half hour at 37°C).

An endothelial cell monolayer is then grown on the surface of the filter coated with the fibronectin or collagen. This is done by methods known in the art. In particular, a composition comprising endothelial cells is added to the coated filter surface, and then cultured under standard growth conditions. Preferably, endothelial cell monolayers that have reached confluence are employed in the assay of the invention; such confluent monolayers prevent media components from diffusing too quickly across the filter during the assay (see *infra*). Endothelial cells suitable for use can be obtained from any *in vivo* source, including but not limited to tissue comprising any vein or artery or microvascular endothelium. Umbilical vein is the most accessible source. Human umbilical vein endothelial cells (HUVECs) are most preferred, and are commercially available (Clonetics Corp., San Diego, CA). Unstimulated endothelium is preferred for use, since stimulated endothelium has yielded higher background levels in the chemotaxis assay of the invention. Procedures for harvesting and culturing endothelial cells are known in the art. For example, HUVECs can be harvested as described by Wheeler et al. (1988, J. Clin. Investigation 82:1211) and cultured as described by Luscinskas et al. (1991, J. Immunol. 146:1617-1625). The endothelial cells are mammalian, including cows, dogs, pigs, and are most preferably human. Human endothelial cells are strongly preferred for use in assaying human lymphocyte chemotaxis.

The lymphocyte chemotaxis assay of the invention is carried out by detecting migration of lymphocytes through the filter containing the EC monolayer on a first surface, wherein migration occurs in the direction from the

first surface of the filter through to the opposite, second surface, wherein a known or suspected chemoattractant is situated below the second surface at increased levels relative to the levels present above the first surface such that the direction of migration is toward the chemoattractant. In a particular embodiment, the filter with the EC monolayer thereon is situated in between two chambers (a "first chamber" and a "second chamber") so as to separate one chamber from the other. The chambers can be formed from various solid phase materials, including but not limited to plastic, glass, quartz, polystyrene, polypropylene, and is most preferably plastic. Preferably, the chambers are separately detachable from the filter, so as to facilitate counting of cells present thereon after chemotaxis has occurred (see *infra*). Optionally, each chamber has one or more ports or inlet means or other openings for injection of solutions. In a preferred embodiment, a Transwell® culture insert such as the insert commercially available from Costar (Cambridge, MA; Transwell® 3422) is employed.

A schematic diagram of an exemplary chemotaxis assay apparatus of the invention is shown in Figure 2. A cluster plate 1 contains a plurality of assay wells, one of which is depicted. The assay well consists of a bottom chamber 2 filled with fluid 3, into which is placed a removable upper chamber 4 containing fluid 5 in which lymphocytes are present. A microporous filter 6 is situated at the interface between the top and bottom chambers, on the upper surface of which filter is a confluent endothelial cell monolayer 7 grown thereon. A cover 8 for the assay well is also provided.

In a specific embodiment, an apparatus of the invention comprises: (a) a filter with a pore size in the range of 4-7 microns; (b) an endothelial cell monolayer grown on the upper surface of said filter; (c) a first chamber having an opening communicating with said endothelial cell monolayer; and (d) a second chamber having an opening communicating with the lower surface of the filter. Preferably, the opening of the second chamber is substantially congruent with the opening of the first chamber.

To one of the chambers of the apparatus ("the first chamber") is added a composition containing lymphocytes. The composition is preferably a

composition that is enriched for lymphocytes and substantially depleted of non-lymphocytic blood cells. In one embodiment, the composition contains a cell population that is greater than 85%, 90%, or most preferably, 95% lymphocytes. Lymphocyte enrichment is carried out by any method known in the art. For example and not by way of limitation, whole blood is collected, an anti-coagulent such as acid citrate is added, peripheral blood mononuclear cells are obtained therefrom by dextran sedimentation and centrifugation on a Ficoll Hypaque cushion, and lymphocytes are enriched and monocytes depleted by collecting non-adherent cells after serial incubations on plastic dishes.

In a specific embodiment (see *infra*) in which migrating lymphocytes are detected or measured by detecting and/or quantitating a label appearing in the other chamber of the apparatus ("the second chamber"), the cells are labeled before placing them in the first chamber. This can be accomplished by any of various methods known in the art, *e.g.*, by fluorescent labeling of the cells, enzymatic labeling (*e.g.*, via an enzyme-tagged antibody to a lymphocyte cell surface marker), etc. In a preferred aspect, cells are fluorescently labeled with fluorescein or a derivative thereof such as 2',7'-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein (BCECF) or calcein (Molecular Probes, Eugene, OR).

Simultaneously, or shortly before or after addition of the cellular composition to the first chamber, a composition comprising or suspected of comprising a lymphocyte chemoattractant is placed inside the second chamber. A "test molecule" as used herein refers to a molecule being tested for the desired activity in promoting or inhibiting lymphocyte chemotaxis, as the case may be (see Sections 5.2 and 5.3, *infra*). A test molecule being tested for its ability to inhibit lymphocyte chemotaxis is preferably placed in the first chamber along with the composition containing lymphocytes when conducting the chemotaxis assay. A test molecule which is being tested for its ability to promote lymphocyte chemotaxis is preferably placed instead of, or (less preferably) in addition to, a known chemoattractant in the second chamber when conducting the chemotaxis assay.

The filter should be situated so as to be in contact with both fluid containing the cellular composition in the first chamber, and fluid containing the chemoattractant/test molecule in the second chamber. Except for added test molecules or cells as stated above, the fluids in the first and second chambers are preferably the same or substantially similar. Additionally, the fluids in the first and second chambers preferably comprise cell culture media, for example, RPMI 1640 (*e.g.*, from Gibco) (or L15) plus M199 (*e.g.*, Gibco) (preferably in a 1:1 ratio). It is important to add some protein such as human serum albumin (HSA), bovine serum albumin (BSA), or fetal calf serum (FCS) to the fluid in both the first and second chambers, to a final concentration in the range of 0.25-1%; the same protein need not be present in both chambers. (Although not intending to be bound by any mechanism, Applicants believe that such proteins aid in the assay of the invention by increasing protein stability and inhibiting nonspecific sticking of cells.) Dilutions of test molecules or chemoattractants are preferably carried out in fluid identical to that present in the chamber to which said molecule or chemoattractant is to be added.

After placement of the cellular composition and chemoattractant composition in the first and second chambers, respectively, the apparatus is incubated to allow any chemotaxis of lymphocytes to take place. Incubation is carried out for a time period in the range of about 3-6 hours, and is most preferably done for 4 hours at about 37°C. In an embodiment where RPMI 1640 medium is employed in one or more of the chambers, incubation is preferably done at 5% CO_2; in an embodiment where L15 medium is employed, incubation at 5% CO_2 is not necessary since L15 can be used in room air.

Following incubation, chemotaxis is detected and/or measured. This is done by detecting and/or measuring the lymphocytes that are present in the second chamber (by virtue of having migrating through the coated filter connecting the first chamber to the second chamber). The detection and/or measurement can be done by any method known in the art. For example, where the cells placed in the top chamber were labeled, the label can be detected and/or measured; for example, cells in the bottom chamber can be counted by

fluorescent microscopy or quantitated in a Pandex fluorescence concentration analyzer. Alternatively, the cells can be retrieved from the second chamber and counted directly under the microscope. Many methods are available to the skilled artisan. Data can be expressed by any convenient method, *e.g.*, as absolute number of cells or as percentage of input cells migrating into the second chamber.

In a preferred aspect, chemotaxis is detected or measured by determining data relative to a control or background level of migration into the second chamber measured in which no chemoattractant or test molecule is placed in the second chamber. An increased number of cells (or percentage of input cells, as the case may be) in the second chamber relative to the background level indicates chemotaxis (or chemokinesis) has occurred. [Chemokinesis can be distinguished from chemotaxis, *e.g.*, by a checkerboard analysis (Zigmond and Hirsch, 1973, J. Exp. Med. 137:387-410)].

5.2. IDENTIFICATION OF PROMOTERS OF LYMPHOCYTE CHEMOTAXIS

Use of transendothelial lymphocyte chemotaxis assay provides a method of screening for promoters of lymphocyte chemotaxis, *e.g.*, lymphocyte chemoattractants. Positive lymphocyte chemotaxis, as detected in the assay, indicates the presence of a lymphocyte chemoattractant.

A lymphocyte chemoattractant is detected by its ability to promote lymphocyte chemotaxis in an assay of the invention, where lymphocyte chemotaxis, in the absence of the chemoattractant molecule, was previously lacking or at decreased levels. Thus, a sample comprising a "test molecule" suspected of having chemoattractant activity is placed in the second chamber of an apparatus of the invention, in a fluid, and an increase in lymphocyte chemotaxis relative to levels of lymphocyte chemotaxis in the absence of the test molecule, or to background levels (baseline levels, with no chemoattractant), indicates that the molecule is a lymphocyte chemoattractant. Molecules to be tested for chemoattractive ability can be any of interest. In a specific embodiment, the chemotaxis assay of the invention can be used to screen natural product or

synthetic chemical libraries (*e.g.*, peptide libraries) to identify novel lymphocyte chemoattractants.

Use of a transendothelial lymphocyte chemotaxis assay of the invention also provides a method of monitoring the purification of a chemoattractant (or, alternatively, of a chemoattraction inhibitor), by observing its activity in the chemotaxis assay at various stages of the purification process. For example, in purifying a chemoattractant, increased purity of a functionally active chemoattractant is confirmed by observing increased chemotaxis in response to samples of the chemoattractant relative to the chemotaxis observed in response to samples of the chemoattractant from earlier stages of the purification process.

5.2.1. LCA, A NOVEL LYMPHOCYTE CHEMOATTRACTANT

In another embodiment, the invention provides a novel substantially purified lymphocyte chemoattractant (hereinafter termed the "LCA," for "lymphocyte chemoattractant"), that is a novelly processed form of MCP-1, having an amino-terminal sequence as follows [beginning at residue 3 of the reported MCP-1 sequence (Yoshimura et al., 1989, J. Exp. Med. 169:1449; Matsushima et al., 1989, J. Exp. Med. 169:1485; Zachariae et al., 1990, J. Exp. Med. 171:2177)]: Asp-Ala-Ile-Asn-Ala-Pro-Val. LCA has chemoattractive activity for T lymphocytes in the assay of the invention. LCA was identified as present in medium conditioned by PHA-activated PBMC, by detecting its chemotactic activity in the assay of lymphocyte chemotaxis provided by the invention. LCA attracts both $CD4^+$ and $CD8^+$ T lymphocytes, and has chemoattractive activity for memory ($CD45RO^+$) but not naive ($CD45RA^+$) T cells. LCA has a molecular weight of about 16,000 daltons, as measured by size exclusion chromatography, and an amino acid sequence as depicted in Figure 18 from amino acid numbers 3-76. The purification of LCA is described by way of example in Section 7, *infra*.

LCA can be obtained from medium conditioned by activated PBMC, preferably PHA-activated PBMC. Purification can be carried out by standard methods known in the art, including but not limited to chromatography

(*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In a preferred aspect, LCA is purified by methods known in the art, such as heparin-Sepharose affinity chromatography followed by size exclusion chromatography and reverse-phase high pressure liquid chromatography, until the chemoattractant is homogenous as determined by SDS-PAGE.

Various assays to monitor purity and confirm identity of LCA can be used; in particular, the lymphocyte chemotaxis assay of the invention is used. Alternatively, or additionally, assays which can be used include but are not limited to those measuring the stimulation by chemoattractants of lymphocyte adhesion to integrin ligands such as ICAM-1, 2, or 3, VCAM-1, fibronectin, laminin, or collagen, immobilized on a substrate. Alternatively, adhesion to fibroblast or endothelial cell monolayers, or after removal of these cells, the underlying extracellular matrix, is measured. Chemoattractants may be mixed with the incubation medium. Alternatively, the chemoattractant is preincubated with proteoglycans, heparin, or chondroitin sulfate coimmobilized with the purified integrin ligands, or with the cells or extracellular matrix, and after washing, a second incubation with lymphocytes carried out. The number of bound lymphocytes is then determined with methods known in the art such as fluorescent dyes.

Chemical synthesis, *e.g.* by use of an automated peptide synthesizer, can also be used to obtain purified LCA.

Recombinant expression according to standard methods, by culturing a host cell containing a recombinant nucleic acid encoding LCA, can also be used to produce LCA. Standard methods for purification of recombinant proteins are well known and can be used.

The chemoattractant we have characterized, LCA, has therapeutic utility as described *infra, e.g.*, for administration in the bloodstream to antagonize lymphocyte accumulation at sites of inflammation and inflammatory disease.

5.2.2. DERIVATIVES AND ANALOGS OF LCA

The invention further provides derivatives (including but not limited to fragments) and analogs of LCA.

The production and use of derivatives and analogs related to LCA are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with the full-length, wild-type LCA protein. Such functional activities include but are not limited to chemoattraction, antigenicity [ability to bind (or compete with an LCA protein for binding) to an anti-LCA antibody], immunogenicity (ability to generate antibody which binds to LCA), and ability to bind (or compete with LCA for binding) to its receptor on a cell (particularly, on a lymphocyte). As one example, such derivatives or analogs which have the desired immunogenicity or antigenicity can be used, for example, in diagnostic immunoassays as described in Section 5.7. Molecules which retain, lack, or inhibit, a desired LCA property, *e.g.*, chemoattraction, binding to its receptor protein, can be used therapeutically as inducers, or inhibitors, as the case may be, of such property and/or its physiological correlates. Derivatives or analogs of LCA can be tested for the desired activity by procedures known in the art, including but not limited to the assays described *infra* in Section 5.2.3.

In particular, LCA derivatives can be made by altering LCA sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. LCA derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a LCA protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine,

proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, proteins consisting of or comprising a fragment of a LCA protein consisting of at least five amino acids of the LCA protein is provided. In other embodiments, the fragment consists of at least 10, 15, 25, 50, or 100 amino acids of the LCA protein. In a specific embodiment, such fragments, as well as derivatives and analogs of LCA, have an amino-terminus as follows: Asp-Ala-Ile-Asn-Ala-Pro-Val.

Derivatives or analogs of LCA include but are not limited to those peptides which are substantially homologous (*e.g.*, greater than 70% identity) to a LCA or a fragment thereof. In specific embodiments, fragments of LCA are at least 15, 30, or 50 amino acids thereof.

The LCA derivatives and analogs of the invention can be produced by various methods known in the art. Also provided by the invention are LCA fragments or other derivatives or analogs which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, etc.

In addition, analogs and derivatives of LCA proteins can be chemically synthesized. For example, a peptide corresponding to a portion of LCA which mediates the desired activity *in vitro* or *in vivo*, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the human LCA protein sequence. Non-classical amino acids include but are not

limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, and Nα-methyl amino acids.

In a specific embodiment, the LCA derivative is a chimeric, or fusion, protein comprising LCA or a fragment thereof (preferably consisting of at least 5 amino acids of the LCA protein) joined at its amino or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.

5.2.3. ASSAYS OF LCA AND ITS DERIVATIVES AND ANALOGS

The functional activity of LCA, and its derivatives and analogs (as well as other chemoattractants identified by the assay of the invention and derivatives and analogs thereof), can be assayed by various methods.

In a preferred embodiment, where one is assaying for chemoattraction or inhibition thereof, the transendothelial lymphocyte chemotaxis assay of the invention is employed. In another embodiment, where one is assaying for the ability to bind or compete with a wild-type LCA protein for binding to anti-LCA protein antibody, various immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting

binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labelled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

The ability to bind to another protein (*e.g.*, a LCA receptor) can be demonstrated by *in vitro* binding assays, noncompetitive or competitive, by methods known in the art.

In another embodiment, physiological correlates of LCA administration to cells can be assayed.

Other methods will be known to the skilled artisan and are within the scope of the invention.

5.3. IDENTIFICATION OF INHIBITORS OF LYMPHOCYTE CHEMOTAXIS

The assays of the invention can be used to identify an inhibitor of lymphocyte chemotaxis, *e.g.*, an antagonist of lymphocyte chemotaxis. Such an inhibitor can be used therapeutically, *e.g.*, as an inhibitor of the inflammatory response by inhibition of lymphocyte extravasation.

Such an inhibitor is detected by its ability to inhibit lymphocyte chemotaxis in an assay of the invention. Thus, in a preferred aspect, such an inhibitor is detected by carrying out the assay of the invention in which: (i) a sample comprising a compound with known lymphocyte chemoattractant activity is placed in the second chamber of the assay apparatus; (ii) a first composition comprising a test molecule suspected of having inhibitory activity is placed in the first chamber; and (iii) a second composition comprising lymphocytes is placed in the first chamber. A decrease in lymphocyte chemotaxis relative to the chemotaxis observed under the same conditions except in the absence of the test molecule, indicates that the molecule is an inhibitor of lymphocyte chemotaxis (and thus has utility as an inhibitor of lymphocyte extravasation).

Molecules to be tested for inhibitory activity can be any of interest, including but not limited to antibodies (preferably monoclonal, most preferably human or humanized monoclonal, or antigen-binding domains thereof),

chemoattractant receptors, integrins, integrin binding partners, peptide antagonists and peptidomimetics, etc. In a specific embodiment, the assay can be used to screen natural product or synthetic chemical libraries (*e.g.*, peptide libraries) to identify antagonists of lymphocyte chemotaxis.

5.4. GENERATION AND USE OF ANTIBODIES TO LYMPHOCYTE CHEMOATTRACTANTS AND CHEMOTAXIS INHIBITORS

According to the invention, the lymphocyte chemoattractants, lymphocyte chemotaxis inhibitors, and derivatives and analogs thereof, provided by the invention, or cells expressing the foregoing may be used as an immunogen to generate antibodies which recognize such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

Various procedures known in the art may be used for the production of polyclonal antibodies. In a particular embodiment, rabbit polyclonal antibodies which bind the molecule of the invention can be obtained. For the production of antibody, various host animals can be immunized by injection with the native molecule, or a synthetic version, or derivative (*e.g.*, fragment) thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human

monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes or portions thereof (*e.g.*, hypervariable regions) from a mouse antibody molecule specific for the desired molecule together with genes from a human antibody molecule (*e.g.*, the constant regions) of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for the molecules, derivatives, or analogs of the invention.

Antibody fragments which contain the idiotype (binding domain) of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked

immunosorbent assay). For example, to select antibodies which recognize a specific domain of a molecule, one may assay generated hybridomas for a product which binds to a fragment of the molecule containing such domain.

The foregoing antibodies can be used therapeutically, as well as in methods known in the art relating to the measurement, localization and activity of the molecules which they recognize, *e.g.*, for imaging these molecules, measuring levels thereof in appropriate physiological samples by immunoassay, etc.

In a specific embodiment, antibodies to LCA are produced. In a particular embodiment, such antibodies are neutralizing (*i.e.*, block the chemoattractive action of LCA). Such antibodies can be identified by various assays, for example, by detecting the ability of the antibody to inhibit lymphocyte chemotaxis toward LCA, when present with LCA in the transendothelial chemotaxis assay of the invention. In another embodiment, such a neutralizing anti-LCA antibody is identified by detecting its ability to block binding of LCA to its lymphocyte cell-surface receptor.

5.5. THERAPEUTIC USES OF LYMPHOCYTE CHEMOATTRACTANTS, CHEMOTAXIS INHIBITORS, AND ANTIBODIES TO THE FOREGOING

The lymphocyte chemoattractants (*e.g.*, LCA) and lymphocyte chemotaxis inhibitors provided by the invention, and analogs and derivatives (including fragments) thereof, and neutralizing antibodies thereto and antibody derivatives, (collectively termed herein "Therapeutics") have use prophylactically and therapeutically in diseases or disorders involving inflammation, diseases or disorders which involve extravasation of lymphocytes (inflammatory and immune disorders), and in cancer.

Lymphocyte chemoattractants, and inhibitors of lymphocyte chemoattraction are particularly important therapeutically, because lymphocytes initiate autoimmune and alloimmune diseases.

It should be noted that chemoattractants can be used as either inhibitors or promoters of the inflammatory response depending on how they are administered. For example, a chemoattractant gradient directing leukocytes

toward a specific tissue is expected to be pro-inflammatory at such tissue, whereas general systemic administration of a chemoattractant is expected to be inhibitory to leukocyte extravasation, since the systemically administered chemoattractant would competitively inhibit leukocyte recognition of chemoattractant gradients directing its migration toward tissues.

As used herein, a "Promoter Therapeutic" shall be construed as a molecule provided by the invention which promotes the inflammatory response, *e.g.*, a chemoattractant (or functional derivative or analog) locally delivered so as to form a gradient directing lymphocytes toward a specific site *in vivo*. As used herein, an "Inhibitor Therapeutic" shall be construed as a molecule provided by the invention which inhibits the inflammatory response, *e.g.*, a systemically administered chemoattractant or functional derivative or analog, a chemotaxis inhibitor, chemoattractant receptor (which can, *e.g.*, bind to chemoattractants and thus competitively inhibit the interaction of chemoattractants with their lymphocyte cell-surface receptors), antibodies to chemoattractants and binding domains of such antibodies, etc.

The invention provides methods of reducing inflammation, and of treating or preventing disorders associated therewith, by administration to a subject of an effective amount of an Inhibitor Therapeutic of the invention. In an alternative embodiment, the invention provides methods of stimulating the inflammatory response, and treating or preventing disorders associated with a deficit in the desired inflammatory response, by administration to a subject of an effective amount of a Promoter Therapeutic of the invention.

The subject is preferably an animal, including but not limited to animals such as cows, pigs, chickens, etc., and is preferably a mammal, and most preferably human.

Diseases and disorders which can be treated by administration of a therapeutically effective amount of an Inhibitor Therapeutic include but are not limited to the following:

Inflammatory arthritis - *e.g.*, rheumatoid arthritis, seronegative spondyloarthritites (Behcets disease, Reiter's syndrome, etc.), juvenile rheumatoid arthritis, vasculitis, psoriatic arthritis, polydermatomyositis.

Systemic lupus erythematosus (SLE).

Asthma.

Inflammatory dermatoses - *e.g.*, psoriasis, dermatitis herpetiformis, eczema, necrotizing and cutaneous vasculitis, bullous diseases.

Inflammatory bowel disease - Crohn's disease and ulcerative colitis.

Tissue damage relating to tissue transplantation.

Other autoimmune disorders. In addition to the autoimmune disorders SLE and rheumatoid arthritis, disorders such as glomerulonephritis, juvenile onset diabetes, multiple sclerosis, allergic conditions, autoimmune thyroiditis, allograft rejection (*e.g.*, rejection of transplanted kidney, heart, pancreas, bowel or liver), and graft-versus-host disease can be treated.

In addition, other diseases and clinical correlates of undesirable inflammatory responses can be treated with Inhibitor Therapeutics of the invention, including but not limited to those associated with hemolytic anemia, blood transfusion, certain hematologic malignancies, inflammatory bowel disease, scleroderma, atherosclerosis, cytokine-induced toxicity, pulmonary granulomas, necrotizing enterocolitis, granulocyte-transfusion-associated syndromes, Reynaud's syndrome, and other central nervous system inflammatory disorders.

Diseases or disorders that can be treated by the Promoter Therapeutics of the invention include but are not limited to immunosuppression (*e.g.*, due to AIDS, cancer chemotherapy, radiation therapy, corticosteroid therapy, or other therapy for autoimmune disease), and congenital immunodeficiencies.

In a specific embodiment, purified chemoattractant is administered into the bloodstream, to inhibit lymphocyte migration into inflammatory sites and thereby inhibit immune diseases associated with lymphocyte and monocyte emigration such as organ transplant rejection, rheumatoid arthritis, inflammatory

bowel disease, psoriasis, asthma, vasculitides, scleroderma, diabetes, and atherosclerosis.

In another specific embodiment, a lymphocyte chemoattractant antagonist, identified by the method described in Section 5.3, is administered by injection into the bloodstream, or into tissue, ingested orally, or by suppository, to inhibit immune diseases associated with lymphocyte and monocyte emigration.

In yet another specific embodiment, a lymphocyte chemoattractant is administered orally or by suppository to attract lymphocytes into the intestine, thus removing them from the body and inhibiting lymphocyte-mediated inflammatory disease.

The invention also provides methods of treating tumors, in particular, malignant tumors, by a method comprising administering a lymphocyte chemoattractant of the invention directly to the site of tumor formation; lymphocytes are thus attracted to the tumor, augmenting leukocyte or immune-mediated regression of the tumor. In a preferred embodiment, a composition comprising a lymphocyte chemoattractant is injected directly into a tumor mass. Tumors which can be thus treated include but are not limited to those of the following types:

Solid tumors
- sarcomas and carcinomas
  - fibrosarcoma
  - myxosarcoma
  - liposarcoma
  - chondrosarcoma
  - osteogenic sarcoma
  - chordoma
  - angiosarcoma
  - endotheliosarcoma
  - lymphangiosarcoma
  - lymphangioendotheliosarcoma
  - synovioma
  - mesothelioma
  - Ewing's tumor
  - leiomyosarcoma
  - rhabdomyosarcoma
  - colon carcinoma
  - pancreatic cancer
  - breast cancer

ovarian cancer
prostate cancer
squamous cell carcinoma
basal cell carcinoma
adenocarcinoma
sweat gland carcinoma
sebaceous gland carcinoma
papillary carcinoma
papillary adenocarcinomas
cystadenocarcinoma
medullary carcinoma
bronchogenic carcinoma
renal cell carcinoma
hepatoma
bile duct carcinoma
choriocarcinoma
seminoma
embryonal carcinoma
Wilms' tumor
cervical cancer
testicular tumor
lung carcinoma
small cell lung carcinoma
bladder carcinoma
epithelial carcinoma
glioma
astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
hemangioblastoma
acoustic neuroma
oligodendroglioma
menangioma
melanoma
neuroblastoma
retinoblastoma

5.5.1. DEMONSTRATION OF THERAPEUTIC UTILITY

Compounds demonstrated to have the desired activity in the apparatuses of the invention can be tested *in vivo* for the desired anti- or pro-inflammatory activity, as the case may be. For example, such compounds can be tested in suitable animal model systems prior to testing in humans, including but

not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. Suitable model systems are also used to demonstrate therapeutic utility (see *infra*).

For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used. For example, an animal model system for rheumatoid arthritis is that consisting of animals of the autoimmune MRL/1 mouse strain (Murphy, E.D. and Roths, J.B., 1978, in Genetic Control of Autoimmune Disease, Rose, N.R., et al., eds., Elsevier/North-Holland, New York, pp. 207-219), that develop a spontaneous rheumatoid arthritis-like disease (Hang et al., 1982, J. Exp. Med. 155:1690-1701).

Other models for various disorders are known in the art and can be employed, such as those described in the following references:

Canella et al., 1990, "Upregulation and coexpression of adhesion molecules correlate with relapsing autoimmune demyelination in the central nervous system," J. Exp. Med. 172:1521-1524.

Cross et al., 1990, "Homing to central nervous system vasculature by antigen-specific lymphocytes. I. Localization of ^{14}C-labeled cells during acute, chronic, and relapsing experimental allergic encephalomyelitis," Lab. Invest. 63:162-170.

García-Vicuña et al., 1992, "VLA family in rheumatoid arthritis: evidence for *in vivo* regulated adhesion of synovial fluid T cells to fibronectin through VLA-5 integrin," Clin. Exp. Immunol. 88:435-441.

Jiang et al., 1992, "Role of $CD8^+$ T cells in murine experimental allergic encephalomyelitis," Science 256:1213-1215.

Kakimoto et al., 1992, "The effect of anti-adhesion molecule antibody on the development of collagen-induced arthritis," Cell. Immunol. 142:326-337.

Keffer et al., 1991, "Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis," EMBO J. 10:4025-4031.

Koh et al., 1992, "Less mortality but more relapses in experimental allergic encephalomyelitis in $CD8^{-/-}$ mice," Science 256:1210-1213.

Mori et al., 1992, "Expression of a transgenic T cell receptor β chain enhances collagen-induced arthritis," J. Exp. Med. 176:381-388.

Printseva et al., 1992, "Various cell types in human atherosclerotic lesions express ICAM-1: further immunocytochemical and immunochemical studies employing monoclonal antibody 10F3," Am. J. Pathol. 140:889-896.

Raine, 1991, "Multiple sclerosis: a pivotal role for the T cell in lesion development," Neuropathol. Appl. Neurobiol. 17:265-274.

Shiozawa et al., 1992, "Destructive arthritis without lymphocyte infiltration in H2-c-fos transgenic mice," J. Immunol. 148:3100-3104.

Sollberg et al., 1992, "Elevated expression of β1 and β2 integrins, intercellular adhesion molecule 1, and endothelial leukocyte adhesion molecule 1 in the skin of patients with systemic sclerosis of recent onset," Arthritis Rheum. 35:290-298.

Thorbecke et al., 1992, "Involvement of endogenous tumor necrosis factor α and transforming growth factor β during induction of collagen type II arthritis in mice," Proc. Natl. Acad. Sci. USA 89:7375-7379.

5.5.2. THERAPEUTIC ADMINISTRATION AND COMPOSITIONS

Various delivery systems are known and can be used to administer a Therapeutic of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, etc. Other methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local, *e.g.*, direct injection at the inflamed joint of someone suffering from rheumatoid arthritis.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic,

and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those

derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers or vials filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

5.6. DIAGNOSTIC UTILITIES

The assay of lymphocyte chemotaxis provided by the present invention has diagnostic utility. Such an assay can be used in the diagnosis of a disease or disorder involving a defect in lymphocyte extravasation, by detecting a deficiency in lymphocyte chemotaxis. Thus, a decrease in lymphocyte chemotaxis observed in the assay of the invention in lymphocytes from a patient relative to

the chemotaxis observed with lymphocytes of a normal or healthy patient or relative to a standard level known to be exhibited by normal or healthy lymphocytes, indicates the presence of a disease or disorder, particularly one involving a deficiency in lymphocyte chemotaxis, *e.g.*, in lymphocyte extravasation. For example, leukocyte adhesion deficiency (Anderson and Springer, 1987, Ann. Rev. Med. 38:175-194) involves an inherited deficiency in the integrins LFA-1, Mac-1, and p150,95, resulting in deficient adherence of granulocytes, monocytes, and lymphoid cells, which should be detectable in a lymphocyte chemotaxis assay of the invention. In addition, diabetes mellitus, granulocytasthenia, and recurrent pyogenic infections have been reported to involve cell adherence defects (see Gallin et al., 1980, Ann. Int. Med. 92:520-538). Genetic defects in chemoattractant receptors, and/or integrins that interfere with extravasation should be capable of detection by the methods of the invention.

The chemoattractants, and antibodies thereto, also have uses in immunoassays, for measuring the amount of chemoattractant or chemoattractant receptors.

6. A TRANSENDOTHELIAL LYMPHOCYTE CHEMOTAXIS ASSAY

As detailed herein, we have developed a novel transendothelial assay for lymphocyte chemotaxis, with high sensitivity and reliability. The conventional chemotaxis assay (Fig. 1A), and its modification for the transendothelial chemotaxis assay (Fig. 1B) were as follows. Leukocytes were prepared by dextran sedimentation and centrifugation on a Ficoll Hypaque cushion (1.077; Sigma) at 1200 xg for 25 min (Dustin and Springer, 1988, J. Cell Biol. 107:321-31). Peripheral blood mononuclear cells (PBMC) were obtained from the Ficoll Hypaque cushion. Granulocytes were collected from the cell pellet and contaminating erythrocytes were removed by hypotonic lysis. Lymphocytes were enriched and monocytes depleted by incubating the PBMC in RPMI-1640 (Gibco Laboratories, Grand Island, NY) with 5% low-endotoxin fetal calf serum (HyClone Laboratories, Logan, UT) on tissue culture-treated plastic dishes for

two 30 min serial incubations at 37°C, 5% CO_2 and collecting the non-adherent cells. During the second enrichment step, leukocytes were fluorescently labeled with 2', 7'-bis-(2-carboxyethyl)-5 (and-6) -carboxyfluorescein (BCECF; Molecular Probes, Inc., Eugene, OR) at 2 μg/ml for 30 min (de Fougerolles et al., 1991, J. Exp. Med. 174:253-267). After incubation, non-adherent cells were collected, washed and resuspended at 5 x 10^6 ml in assay medium, a 1:1 mixture of RPMI-1640/M199 (Gibco) plus 1% human serum albumin (HSA). For monocyte chemotaxis experiments, monocytes were recovered from the dishes by treatment with ethylene diamine tetracetic acid (EDTA) and resuspended as described for lymphocytes. Neutrophils were labeled in suspension with BCECF for 30 min at 37°C and resuspended in assay media at 1.5 x 10^6 cells/ml.

Conventional chemotaxis assays utilized Transwell® culture inserts (Transwell® 3422, Costar, Cambridge, MA) with 6.5 mm diameter tissue culture-treated, polycarbonate membranes (Capsoni et al., 1989, J. Immunol. Methods 120:125-31; Casale and Abbas, 1990, Am. J. Physiol. 258:C639-47; Partsch et al., 1989, Z. Rheumatol. 48:123-28). Filters with varying pore sizes were used, depending on the cell type being assayed; 3 μm pores were used to measure neutrophil chemotaxis, 5 μm for monocyte chemotaxis and 5 and 8 μm for lymphocyte chemotaxis. In some experiments, Transwells® were pretreated with ICAM-1 (1:20 dilution of purified transmembrane form), collagen (40 μg/ml, Organogenesis, Inc.), or fibronectin (10 μg/ml, Telios, Inc.); this had no significant effect on conventional chemotaxis assays. Chemotactic factors or control media were added, in duplicate or triplicate, to 24-well tissue culture plate(s) in 600 μl final volume. Fluorescently labeled cells were added to the top chamber of each Transwell® in a final volume of 100 μl assay medium. In some experiments, a 1:20 dilution of cells was added to a well containing only a bottom chamber with media. This allowed measurement of the input number of cells. The assay plate(s) was incubated for 4 hr at 37°C, 5% CO_2. Following incubation, the Transwells® were removed and the cells in each bottom well were counted by fluorescent microscopy. Cells in the bottom chamber were resuspended by vigorous pipetting and then let settle for about 45-60 minutes until

all cells had settled. Five fields of cells were counted per well, using an ocular grid at 100X magnification including the input well, and the number of cells per grid was averaged and presented as percent of input cells migrating to the bottom chamber of the Transwell®. In some experiments, data were presented as "number of cells per grid" and no attempt was made to calculate percent of migrating cells. All samples were run in duplicate or triplicate.

Transendothelial chemotaxis assays utilized human umbilical vein endothelial cells (HUVECs) in passage one to four, cultured on 6.5 mm diameter transwell culture filters in which the tissue culture-treated, polycarbonate membrane (8 μm pore size) had first been treated for at least 0.5 hr at 37°C with type I collagen (40 μg/ml, Organogenesis, Inc., Canton, MA). HUVECs were added to the insert's collagen-coated membrane surface at a concentration of 50,000-100,000 cells/well and were cultured for 5-8 days in M199 medium (Gibco) supplemented with 10-20% low endotoxin fetal calf serum (HyClone), 2 mM L-glutamine, 5 μg/ml gentamicin, 25 mM HEPES, 100 μg/ml porcine intestinal heparin (Sigma), and 100 μg/ml endothelial cell growth factor (Biomedical Technologies, Stoneham, MA) at 37°C, 5% CO_2. Media was changed every 2 days and confluence was judged by microscopic examination after staining one or more of the monolayers with Wright/Giemsa or the fluorescent label BCECF. Transwells® with unstained confluent endothelial monolayers were then used for assay in the same way as for the conventional chemotaxis assay as described above. A control was usually done to monitor diffusion from the bottom chamber into the top chamber, in order to ensure that components in the bottom chamber did not diffuse too quickly into the top chamber over the course of incubation. This was done by adding fluorescently labeled dextran to the bottom chamber of a Transwell® apparatus, and conducting the assay in the same way as described above. Usually a four- to nine-fold greater level of dextran was present in the bottom chamber relative to the top chamber at the end of the 4 hr incubation time.

We discovered that lymphocytes behave differently than monocytes and neutrophils in chemotaxis chambers. Neutrophils migrated vigorously to the

formylpeptide f-Met-Leu-Phe and IL-8 (Fig. 3A), and monocytes responded well to f-Met-Leu-Phe and much less so to RANTES (Fig. 3B). The response is readily measurable by counting the number of cells that appear in the bottom chamber. Lymphocytes migrated through polycarbonate filters with 5 μm pores but with a very low signal to noise ratio relative to that obtained in the transendothelial assay of the invention (not shown). A high percentage of lymphocytes (30-40%) migrated or fell through polycarbonate filters with 8 μm pores, whether or not chemoattractant was added (Fig. 4A). The same 8 μm filters in the same geometry are used by other investigators to assay for chemotaxis by counting the number of cells that migrate through the filter and stick to the underside (Schall et al., 1990, Nature 347:669-71). However, our results show that these must be numerically a very small percentage of the cells that fall or migrate through the filter. Therefore, these may be assays of sticking rather than chemotaxis.

We have thus developed an excellent lymphocyte chemotaxis assay by culturing endothelium on microporous filters coated with collagen or fibronectin and assaying lymphocyte transmigration toward chemoattractants (Fig. 1B, Fig. 4B). A signal to noise ratio of greater than 10 was common, and a response was elicited to supernatants from the mixed lymphocyte reaction (MLR) or phytohemagglutinin (PHA)-stimulated lymphocytes (Fig. 4B). Mixed lymphocyte reaction (MLR) supernatant was generated as described by Bach and Voynow (Bach and Voynow, 1966, Science 153:545-47). Briefly, peripheral blood was drawn from two individuals and prepared as described above. PBMC from one donor were resuspended at 1 x 10^7 cells/ml and treated with 50 μg/ml mitomycin C (Sigma) for 20 min at 37°C (stimulator cells). Following mitomycin C treatment, stimulator cells were washed 3X and resuspended at 2 x 10^6 cells/ml. Meanwhile cells from the second donor (responder cells) were enriched for lymphocytes as described above but without the BCECF labeling. Non-adherent cells were collected and resuspended at 2 x 10^6 cells/ml. Responder and stimulator cells were combined at a 1:1 ratio in RPMI-1640 supplemented with 5% FCS, 2 mM L-glutamine, and 50 μg/ml gentamicin such

that the final concentration of the cells was 4 x 10^6 cells/ml. Cultures were incubated at 37°C, 5% CO_2 for 6 days after which supernatant was collected, filtered, and stored at 4°C. For supernatant from PHA-stimulated PBMC, PBMC were obtained from a leukopak (Children's Hospital, Boston, MA) by the same methods. PBMC were resuspended at 2-4 x 10^6 cells/ml in serum-free media supplemented with 2 mM L-glutamine, 10 mM HEPES, 50 μg/ml gentamicin, 1 μg/ml indomethacin, 3 mM lithium chloride, 50 μM hydroxyurea, and 2.5 μg/ml PHA-P. Cultures were incubated at 37°C, 5% CO_2 for 3 days after which supernatant was collected, filtered, and stored at -70°C.

The results with the transendothelial chemotaxis assays (Fig. 4B) were in sharp contrast to assays with microporous membranes alone (Fig. 4A). The transendothelial chemotaxis assay system accurately recapitulates lymphocyte emigration from blood vessels toward chemoattractants *in vivo*, in which cells migrate through endothelium and basement membrane into tissues. These assay chambers may thus be called a type of "artificial vessel construct."

Other artificial vessel constructs, composed of endothelial cells cultured on matrices of type I collagen, had previously been used to study neutrophil migration toward the chemoattractant IL-8 (Huber et al., 1991, Science 254:99-102). When tested with IL-8 contained in conditioned media from stimulated ECs, neutrophils migrated similarly, and there was a similar signal-to-noise ratio, on collagen matrix plus endothelium and on matrix alone. There was no indication that the assay would be improved for other cell types by adding endothelium, as we have surprisingly shown to be the case for lymphocytes. Indeed, the expectation would have been that neutrophils and lymphocytes would behave similarly.

Lymphocyte migration across endothelium into collagen gels has previously been reported (Kavanaugh et al., 1991, J. Immunol. 146:4149-56; Masuyama et al., 1992, J. Immunol. 148:1367-74). In this system, the endothelium is cultured on a collagen gel formed on a culture dish or well for several days, then lymphocytes are added. However, this is an assay of migration and there is no evidence that chemotaxis is involved; rather, it appears to assay

for a migratory subset of lymphocytes. There is no provision for addition of a chemoattractant, and note that, as described below, we find no evidence that endothelium releases a chemoattractant for lymphocytes. Furthermore, lymphocytes migrate into collagen gels even in the absence of an endothelial cell monolayer.

Endothelium may have several properties that enable lymphocyte chemotaxis to be successfully measured. There may be specialized molecules on endothelium such as proteoglycans that retain chemoattractants for presentation to lymphocyte chemoattractant receptors; chemoattractive cytokines such as IL-8 and MCP-1 have heparin binding sites that would enable binding to proteoglycans (Oppenheim et al., 1991, Ann. Rev. Immunol. 9:617-48). LCA also seems to have heparin binding sites as demonstrated by its binding to Heparin-Sepharose during purification (Fig. 10). There could also be specialized endothelial cell surface projections such as microfimbrae that bind to lymphocytes and somehow aid chemotaxis. Finally, endothelial cells form intercellular junctions that impede diffusion of solutes.

We have found that the endothelial cell monolayer significantly retards the diffusion of macromolecules such as fluorescein isothiocyanate (FITC)-immunoglobulin and FITC-Dextran (molecular weight 4.4 kD) (Fig. 5). In the absence of endothelium, FITC-Dextran diffuses across filters with 8 μm pores almost to equilibrium in 4 hours. Thus, the gradient at the filter is almost completely dissipated. By contrast, the retardation of diffusion by endothelium results in a sharp gradient, indeed a large step in concentration, immediately at the endothelium. One possible reason for the effectiveness of our assay is that lymphocytes may require steeper chemoattractant gradients that monocytes or neutrophils.

The chemotaxis assay shows excellent dose-response characteristics (Fig. 6). The response was maximal to MLR supernatant that was undiluted (neat) or diluted 1:2, and fell off at greater dilution. This concentration-dependence demonstrates the utility of the assay for measuring chemoattractant in

different separations or chromographic fractions during purification and thus in obtaining highly purified material.

We examined several potential chemoattractant sources and lymphokines that have been reported using other assays to have lymphocyte chemoattractant activity, in the transendothelial chemotaxis assay (Fig. 7). We found that IL-1, IL-2, and IL-4 were without effect, in contrast to results previously reported in the less physiologically relevant naked filter chemotaxis assay (Berman et al., 1988, Immunol. Invest. 17:625-77). Endothelial cells failed to release chemoattractants for lymphocytes after 20 hours stimulation with the cytokines IL-4 or IL-1. By contrast, MLR supernatant was highly active as a positive control. There was a much lower response to f Met-Leu-Phe, due to a small percentage of monocytes in the mononuclear cell preparation, as described below.

We examined the effects of known chemoattractive cytokines, and the phenotype of the migrating cells (Fig. 8). The cell preparation routinely used in our assays is mononuclear cells purified by Ficoll-Hypaque gradients. These cells are depleted of monocytes by two cycles of adherence to tissue-culture treated dished. The cells are >90% lymphocytes, and contain a small percentage (<10%) of monocytes. Transendothelial chemotaxis was carried out in large (24.5 mm) Transwells®. Migrating cells were stained with mAb to CD3 for T lymphocytes and CD14 for monocytes and the number of cells in each population was quantitated. The MLR supernatant attracted $CD3^+$ T lymphocytes and almost no $CD14^+$ monocytes. By contrast, fMLP was selective for monocytes. MCP-1, MIP-1α, and RANTES, which all belong to the chemokine protein family (Oppenheim et al., 1991, Annu. Rev. Immunol. 9:617-48), showed little if any effect on T lymphocytes. The chemokine IL-8 was also without effect on T lymphocytes (not shown). MCP-1α and RANTES had significant monocyte chemoattractive activity. In conclusion, previously known cytokines or lymphokines cannot account for the lymphocyte chemoattractive activity found in MLR supernatants, and this activity is highly specific for lymphocytes.

We also undertook an analysis of chemotactic versus chemokinetic activity in MLR supernatant. Chemokinesis is defined as stimulated motility that is random in direction, whereas chemotaxis is directional towards the stimulus. A checkerboard analysis, applying the method described by Zigmond and Hirsch (Zigmond and Hirsch, 1973, J. Exp. Med. 137:387-410) to our transendothelial chemotaxis assay, was used to differentiate the specific versus random migration of lymphocytes responding to MLR supernatant. The activity we have defined is directional, because "checkerboard assays" show that lymphocytes migrate when chemoattractant is present in the bottom chamber and not in the top, but that migration falls off as chemoattractant is added to the top chamber (Table 1).

Table 1

CHEMOTACTIC vs. CHEMOKINETIC ACTIVITY OF MLR SUPERNATANT***

TOP / BOTTOM	MEDIA	1:4*	NEAT**
MEDIA	0.10%	0.13%	0.78%
1:4*	3.10%	1.22%	0.32%
NEAT**	7.12%	3.14%	1.15%

* MLR supernatant diluted 1:4 with media was used.
** NEAT = undiluted MLR supernatant
*** Data is presented as percentage of input lymphocytes which migrate into the bottom chamber.

This finding in our artificial vessel construct demonstrates that adding chemoattractant to the vessel side inhibits chemotaxis. Thus, administering chemoattractant into the bloodstream of patients should block chemotaxis of

lymphocytes into sites of inflammatory disease by inhibiting the response toward chemoattractants present in the diseased tissue.

We have thus far examined responses to supernatants of several types of cultured leukocytes and cell lines. PHA-stimulated lymphocytes consistently release the same or greater activity than MLR cultures in serum-free medium. The migrating cells have the same phenotype of $CD3^+$ lymphocytes as found for MLR supernatant (Fig. 9).

7. CHARACTERIZATION OF A NOVEL LYMPHOCYTE CHEMOATTRACTANT

The lymphocyte chemoattractant (termed "LCA") we identified as present in media conditioned by PHA-activated PBMC was concentrated and then purified and characterized as described below. Using the chemotaxis assay of the invention, we purified to homogeneity and sequenced the major lymphocyte chemoattractant secreted by mitogen-stimulated PBMC. Surprisingly, we found it to be MCP-1, a known monocyte chemotactic factor, and a variant thereof with a novelly processed amino-terminus. MCP-1 is the prototypic member of the β chemokine family which has previously been thought to be chemoattractive only for monocytes.

As described below, the chemoattractant was purified from supernatants of mitogen-stimulated PBMC by heparin-Sepharose, size exclusion, and HPLC chromatography. Amino acid sequence analysis revealed it to be MCP-1 and a variant thereof with a novelly-processed amino-terminus. We showed that recombinant MCP-1 is chemoattractive for purified T lymphocytes and for $CD3^+$ lymphocytes in PBL preparations. Furthermore, we showed that the majority of T lymphocyte chemotactic activity in mitogen-stimulated PBMC supernatants is neutralized by antibody to MCP-1. Phenotyping of chemoattracted T lymphocytes shows that they are an activated, memory subset. The T lymphocyte response to MCP-1 is chemotactic, rather than chemokinetic, as demonstrated in a checkerboard assay. Furthermore, the response to MCP-1 by T lymphocytes is not dependent on the endothelium present in our chemotaxis assay system, since the response can be duplicated on bare filters. We conclude

that MCP-1 is capable of acting as a potent T lymphocyte, as well as monocyte, chemoattractant and this may help explain why monocytes and T lymphocytes of the memory subset are always found together at sites of antigen-induced inflammation.

7.1. MATERIALS AND METHODS

7.1.1. PREPARATION OF PERIPHERAL BLOOD LYMPHOCYTES AND PURIFIED T CELLS

PBMC were obtained by dextran sedimentation and Ficoll-Hypaque (1.077; Sigma, St. Louis, MO) centrifugation (Dustin and Springer, 1988, J. Cell Biol. 107:321). Monocytes were depleted by incubating the PBMC on tissue culture-treated plastic Petri dishes for two 30 min serial incubations at 37°C, 5% CO_2, to obtain PBL. During the second monocyte depletion step, PBL were fluorescently labeled with 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF; Molecular Probes, Inc., Eugene, OR) at 0.5 μg/ml for 30 min (de Fougerolles et al., 1991, J. Exp. Med. 174:253) to help discriminate lymphocytes from endothelial cell debris in the bottom chamber following transendothelial migration. PBL were washed and resuspended at 5 x 10^6/ml in assay medium, a 1:1 mixture of RPMI-1640:M199 plus 0.25% human serum albumin. Preparations of PBL typically contained less than 5% monocytes as determined by flow cytometry.

T cells were purified using the Magnetic Cell Separation (MACS) protocol developed by Miltenyi Biotec (Sunnyvale, CA) (Miltenyi et al., 1990, Cytometry 11:231; Abts et al., 1989, J. Immunol. Methods 125:19). Briefly, PBMC prepared as described above were incubated for 30 min on ice with 5 μg/ml each of the antibodies, MY4 (Coulter Cytometry, Hialeah, FL; Griffin et al., 1981, J. Clin. Invest. 69:932), OKM1 (Wright et al., 1983, Proc. Natl. Acad. Sci. USA 80:5699), B1 (Coulter; Nadler et al., 1981, J. Clin. Invest. 67:134), and 3G8 (Fleit et al., 1982, Proc. Natl. Acad. Sci. USA 79:3275), to deplete monocytes, B cells, and natural killer cells, respectively. Following incubation, cells were washed and incubated with 20 μl of MACS rat anti-mouse IgG1 and IgG2a+2b microbeads (Miltenyi Biotec, distributed by Becton

Dickinson, San Jose, CA) per 10^7 cells for 20 min on ice. Following the incubation, cells were loaded onto a pre-equilibrated MACS magnetic separation column coupled to the MACS magnet system. Flow cytometry showed purified T cells contained <1% monocytes, B cells or natural killer cells. Remaining T cells were stained with BCECF, washed and resuspended in assay media as described for PBL.

7.1.2. TRANSENDOTHELIAL CHEMOTAXIS ASSAY

Isolation and culture of human umbilical vein endothelial cells (HUVEC) was performed as previously described (Gimbrone, 1976, Prog. Hemost. Thromb. 3:1). HUVEC in passage one to four were cultured on collagen-coated, 6.5 mm diameter Transwell® culture inserts (COSTAR, Cambridge, MA) with 8 μm pore size (or 5 μm where noted). Chemotactic factors or control media were added in duplicate to 24-well tissue culture plate(s) in 600 μl final volume. All chemoattractants were diluted in assay media. HUVEC-coated Transwells® were inserted into each well and fluorescently labeled PBL or T cells were added to the top chamber in a final volume of 100 μl. A 1/20 dilution of these cells added to a well containing media alone, but without a Transwell® insert, served as a measure of the input number of cells. The assay plate(s) was incubated for 4 hr at 37°C, 5% CO_2. Following incubation, the Transwells® were removed, the cells in each well bottom were vigorously resuspended, allowed to settle, and counted by fluorescent microscopy. Four 10 x 10 grids (0.1 mm/grid) were counted per well. The number of cells per grid was averaged and presented as percent of input cells migrating to the bottom chamber of the Transwell®. All samples were run in duplicate and each experiment has been repeated a minimum of three times with similar results. In each experiment, the ability of the endothelial cell monolayer to act as a barrier against the upward diffusion of low molecular weight substances was assessed. A checkerboard analysis, applying the method described by Zigmond and Hirsch (1973, J. Exp. Med. 137:387) to our transendothelial chemotaxis assay, was used to differentiate the specific versus random migration of lymphocytes responding to

recombinant MCP-1. A neutralizing, polyclonal rabbit anti-human MCP-1 antibody (Genzyme, Cambridge, MA) and normal rabbit IgG (R&D Systems, Minneapolis, MN) were used in some transendothelial chemotaxis assays. Purified recombinant MCP-1, expressed in *E. coli*, was purchased from Preprotech, Inc. (Rocky Hill, NJ) and purified recombinant MCP-1, expressed transiently in COS cells, was a kind gift of Dr. B. Rollins (Dana Farber, Boston, MA).

7.1.3. GENERATION OF CHEMOATTRACTANT SUPERNATANT

Peripheral blood leukopaks, obtained as a product of platelet pheresis (Children's Hospital, Boston, MA) were subjected to Ficoll-Hypaque centrifugation as described above to obtain PBMC. PBMC were washed and resuspended at 2-4 x 10^6 cells/ml in serum-free X-VIVO media (M.A. Whittaker, Walkersville, MD) supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin, 1 μg/ml indomethacin, 3 mM lithium chloride, 50 μM hydroxyurea, and 2.5 μg/ml PHA-P (Sigma, St. Louis, MO). After 3 days at 37°C, culture supernatant (termed PHA supernatant) was collected, centrifuged, filtered, and stored at -70°C.

7.1.4. HEPARIN SEPHAROSE AFFINITY CHROMATOGRAPHY

PHA supernatant (4 to 6 liters) was adsorbed to a 40-ml heparin-Sepharose CL-6B column (Pharmacia) at a flow rate of 0.7 ml/min. The column was washed with 0.01 M Tris-HCl pH 7.5, 0.15 M NaCl and protein was eluted using a 300 ml salt gradient from 0.15 to 1.5 M NaCl in 0.01 M Tris-HCl pH 7.5. The elution rate was 0.2 ml/min and 5.0 ml fractions were collected. Salt concentration was determined by conductivity.

7.1.5. SIZE EXCLUSION CHROMATOGRAPHY

Pooled fractions from heparin-Sepharose chromatography were concentrated approximately 20x using Amicon (Beverly, MA) Centriprep concentrators with molecular weight cut-offs of 3,000 daltons and applied to a

125-ml Sephadex G-75 column (Pharmacia). The column was run at a flow rate of 0.07 ml/min and 1.25 ml fractions were collected.

7.1.6. REVERSE PHASE HPLC

Active fractions from G-75 chromatography were concentrated approximately 40x as described above and applied to a 4.6 x 150 mm C4 reverse phase column (5 μm; Vydac), equilibrated with 0.1% trifluoroacetic acid (TFA) in water. A three-step gradient was programmed with 0-25% acetonitrile with 1% change in acetonitrile concentration per minute, 25-45% acetonitrile with 0.2% change/minute, and 45-80% acetonitrile with 1% change/minute. Acetonitrile solutions were vol/vol in water containing 0.1% TFA. Flow rate was 1 ml/min and all protein peaks were collected individually.

7.1.7. SEQUENCE ANALYSIS

HPLC fractions were subjected to N-terminal sequence analysis or proteolytic digestion in solution, followed by HPLC separation and sequencing, as has been described previously (Lane et al., 1991, J. Prot. Chem. 10:151). For matrix-assisted laser desorption mass spectrometry, 0.5 μl (approximately 0.1 to 1.0 pmol) of each peptide fraction was mixed with 0.5 μl of 10 mg/ml α-cyano-4-hydroxycinnamic acid and spotted onto a stainless steel target. The mass spectrum was obtained by summing the data from 5 to 40 pulses of a 337 nm nitrogen laser on a Finnigan Lasermat (Hemel Hempstead, UK) time-of-flight mass spectrometer.

7.1.8. PROTEIN DETERMINATIONS

Protein concentrations for fractions at each step of purification were determined by measuring absorbance at 280 nm and 310 nm. These values were then used in the following formula to obtain a value of mg/ml of protein: A_{280}-A_{310}/1.5. PHA supernatant and heparin-Sepharose-purified material were dialyzed against PBS prior to measuring absorbances.

7.1.9. FLOW CYTOMETRY OF MIGRATED LEUKOCYTES

The transendothelial chemotaxis assay was performed as described above but with the following modifications. Endothelial cells were grown on 24.5 mm diameter, 8 μm Transwell® filters. Recombinant MCP-1 was added to the bottom chambers in a final volume of 2 ml and 1 ml of non-BCECF-labeled PBL at 5-7 x 10^6 cells/ml were added to the top chamber. Following incubation, the migrated cells were harvested by vigorous pipetting (with confirmation by microscopy to assure that all adherent cells were harvested), washed, and resuspended at a concentration of approximately 10^7 cells/ml in L-15 media (Gibco Laboratories, Grand Island, NY) with 2% FCS. For indirect immunofluorescence analyses, cells were stained with the mouse anti-human antibodies MY4, anti-CD3 (Coulter; Reinherz and Schlossman, 1980, Cell 19:821), or X63 IgG1 myeloma as a negative control, then with FITC-labeled goat anti-mouse heavy and light chain IgG (Zymed Immunochemicals, So. San Francisco, CA) as previously described (Hibbs et al., 1990, J. Clin. Invest. 85:674). All volumes were scaled down to 10 μl and cells were washed with L-15/2%FCS. For three color fluorescence, mouse anti-human monoclonal antibodies directly conjugated to fluorochromes were used; anti-CD3 (Caltag, So. San Francisco, CA); UCHL-1 to CD45RO (Serotec, distributed by Harlan Bioproducts, Indianapolis, IN; Akbar et al., 1988, J. Immunol. 140:2171); MY4 to CD14, T4 to CD4 (Reinherz and Schlossman, 1980, Cell 19:821), T8 to CD8 (id.), B1 to CD20, 2H4 to CD45RA (Morimoto et al., 1985, J. Immunol. 134:3762), Ta1 to CD26 (Fox et al., 1984, J. Immunol. 133:1250); 4B4 to CD29 (Morimoto et al., 1985, J. Immunol. 134:3762), and mouse IgG (Coulter Cytometry); anti-Leu-8 to L-selectin (Kansas et al., 1985, J. Immunol. 134:2995), and anti-Leu-11 to CD16 (Phillips and Babcock, 1983, Immunol. Letters 6:143) (Becton Dickinson). All antibodies were coupled to FITC with the exceptions of anti-CD3 which was coupled to R-phycoerythrin (PE)-Cy5 tandem conjugate (TC) and MY4 which was coupled to either FITC or PE. Additionally, biotinylated C4A9, an antibody to an endothelial antigen (L. Klickstein, unpublished results) followed by PE-coupled streptavidin (Gibco) was used to

identify endothelial cell debris in some experiments. Cells were gated based on their forward versus 90° scatter profile. PE-conjugated antibodies specific to monocyte and endothelial cell antigens were added to confirm that all gated cells were lymphocytes. Antibody staining was according to manufacturers' instructions. Three color fluorescence was analyzed using a Becton Dickinson FACS Scan.

7.2. RESULTS

7.2.1. PURIFICATION OF A LYMPHOCYTE TRANSENDOTHELIAL CHEMOATTRACTANT

A supernatant from PBMC stimulated for 3 days with the mitogen PHA-P was screened, with the transendothelial chemotaxis assay, for potential lymphocyte chemotactic activity. Typically, the lymphocyte chemotactic response to this PHA supernatant was 10x over the response to media control or to PHA-P alone. The lymphocyte chemotactic factor was subjected to purification using the transendothelial chemotaxis assay to identify active fractions. Lymphocyte chemotactic activity was adsorbed to heparin-Sepharose and eluted in one major peak between 0.5 and 0.6 M NaCl (Fig. 10). This led to a 24x increase in specific activity over the starting supernatant and a recovery of 31% of the activity (Table 2).

Table 2
Purification of MCP-1

	Protein (mg)[a]	Volume (ml)	Activity (units)[b]	Specific Activity (units/mg)
PHA Supernatant	2136	5400	1.6×10^6	749
Heparin-Sepharose	24	54	4.9×10^5	1.8×10^4
G-75 Sephadex	1.5	15.5	1.1×10^5	7.0×10^4
Reverse Phase HPLC	0.014[c]	--	--	--

[a] Protein concentrations were determined by measuring absorbance as described in Materials and Methods.
[b] Activity of 1U/ml was defined as the reciprocal of the dilution at which 50% of the maximal chemotactic response was obtained within a single assay.
[c] Protein concentration was determined by amino acid analysis.

Little to no activity was detected in either the column flow-through or the column wash, despite the fact that the majority of starting protein was found in these fractions. Active fractions were pooled, concentrated, and applied to a Sephadex G-75 size exclusion column (Fig. 11). In this and other size exclusion purifications, the center of the peak was found consistently to correspond to a molecular mass of 16 kD. Although there is a suggestion of two peaks of activity in Fig. 11, this was not a consistent finding. The specific activity of the chemoattractant was increased approximately 4x in this step with a 22% recovery (Table 2). The active fractions were pooled, concentrated, and applied to a C_4 reverse phase HPLC column. The lymphocyte chemotactic activity consistently eluted in 28-29% acetonitrile corresponding to a prominent protein doublet (Fig. 12). Fractions eluting between 25-35% acetonitrile were assayed for chemoattractant activity because previous reverse phase chromatography experiments had localized all of the chemotactic activity to this region (data not

shown). Because all remaining protein was subjected to sequencing, no attempts were made to titrate the activity and no specific activity was determined at this step.

The two active protein peaks, along with some of the other prominent, non-active peaks were subjected to laser desorption mass spectrometry and amino acid analysis followed by N-terminal sequence analysis. The molecular masses of the two active peaks were 9336 and 8657 as determined by laser desorption mass spectrometry. Amino acid analysis of the peaks showed that they contained 440 pmol and 424 pmol of protein. In the first purification, the N-termini of both active peaks were blocked. Therefore, these fractions were subjected to proteolytic digestion, followed by reverse phase HPLC separation and microsequencing of the resulting peptides. The amino acid sequences obtained (Table 3) were found to be identical to the sequence of human MCP-1 (Yoshimura et al., 1989, J. Exp. Med. 169:1449; Matsushima et al., 1989, J. Exp. Med. 169:1485; Zachariae et al., 1990, J. Exp. Med. 171:2177).

Table 3
Comparison of Sequenced Peptides with MCP-1 Sequence*

N-Terminus	D A I N A P V
hMCP-1 (pos. 3-9)	D A I N A P V
Peptide 2A-CT17	X V Q D S M D H L D K Q T Q - P K T
Peptide 2A-CT28	E I C A D P K Q(K)X V Q(D)
Peptide 2B-CT29	[W]V Q D S M D H L D K Q T Q T P K[T]
Peptide 2B-CT40	E I C A D P K Q K X V(Q)D X M X(H)
hMCP-1 (pos. 49-76)	K E I C A D P K Q K W V Q D S M D H L D K Q T Q T P K T

* Amino acid sequence (one-letter symbols) obtained from the N-terminus or tryptic digest peptides of the purified lymphocyte chemoattractant peak from HPLC are compared to the human MCP-1 sequence (Yoshimura et al., 1989, J. Exp. Med. 169:1449; Matsushima et al., 1989, J. Exp. Med. 169:1485; Zachariae et al., 1990, J. Exp. Med. 171:2177). Confidence code for sequenced amino acids: A = High; [A] = Probable/Reasonable; (A) = Possible/Low; X = No residue identified.

In a second purification, N-terminal sequence analysis of the active peak yielded an MCP-1 N-terminal sequence that began with residue 3, corresponding to a novelly processed form of MCP-1 (Table 3). Most of the MCP-1 had this novelly processed N-terminus, since application of 40 pmol yielded 23 pmol of the N-terminal aspartic acid residue. Sequencing of the other major peak from HPLC, eluting at 32% acetonitrile, showed that it was IL-8. This peak was consistently inactive in the transendothelial lymphocyte chemotaxis assay.

7.2.2. RECOMBINANT MCP-1 IS A T CELL CHEMOATTRACTANT

To further confirm that MCP-1 is a lymphocyte chemoattractant, purified prokaryotically-expressed, recombinant human MCP-1 was tested in our transendothelial chemotaxis assay, using both peripheral blood lymphocytes and purified T cells as the migrating cells. fLMP was included as a reference chemoattractant for monocyte migration and concentrated PHA supernatant was included as a positive control. Both PBL and T cells migrated to MCP-1 (Fig. 13). The PBL population contains some monocytes that respond to the fMLP control, although there is more migration to MCP-1. However, purified T cells migrate to MCP-1, but not to fMLP. T cell chemotaxis to recombinant MCP-1 was shown to be dose dependent (Fig. 14). Optimal chemotaxis was seen at 50 ng/ml and a good T cell response was seen at 20 ng/ml, the concentration recommended for optimal monocyte chemotaxis. We found that eukaryotic and prokaryotic recombinant MCP-1 gave similar dose response curves for T cell chemotaxis (data not shown).

Purified T cells were subjected to checkerboard analysis to examine whether MCP-1-induced migration is chemotactic or chemokinetic (Table 4).

Table 4
Checkerboard Analysis of T Cell Migration to MCP-1*

BOTTOM	TOP: 50 ng/ml	20 ng/ml	10 ng/ml	0 ng/ml
		migration (%)		
50 ng/ml	0.12 (±0.04)	0.56 (±0.03)	1.17 (±0.00)	3.69 (±0.96)
20 ng/ml	0.10 (±0.01)	0.16 (±0.06)	0.68 (±0.06)	1.06 (±0.08)
10 ng/ml	0.01 (±0.00)	0.11 (±0.07)	0.09 (±0.04)	0.53 (±0.01)
0 ng/ml	0.01 (±0.00)	0.13 (±0.00)	0.17 (±0.04)	0.11 (±0.08)

* Purified T lymphocytes were assayed for transendothelial chemotaxis, with the indicated concentrations of recombinant MCP-1 in the top and bottom compartment of the wells. Values in parentheses show range of duplicates. The experiment shown is representative of three similar experiments.

When the gradient of MCP-1 between the top and bottom chambers was equal or if there was a greater concentration of MCP-1 in the top chamber compared to the bottom, lymphocyte migration was at background levels. In contrast, when a greater concentration of MCP-1 was in the bottom chamber, migration was approximately 10-30x over background migration. Thus, the T cell response to MCP-1 is due to directed migration rather than to increased random migration.

Purification of MCP-1 from PHA-supernatant as a lymphocyte chemoattractant does not rule out the presence of other lymphocyte chemoattractant activities in PHA-supernatant. Polyclonal rabbit anti-human MCP-1 antibody consistently neutralized the majority, but not all, of the T cell chemotactic activity present in PHA-supernatant (Fig. 15). In contrast, this MCP-1 antibody was able to completely neutralize the T cell response to recombinant MCP-1.

7.2.3. ENDOTHELIAL CELLS ARE NOT NECESSARY FOR T CELL CHEMOTAXIS TO MCP-1

To address the question of whether chemotaxis of T cells to MCP-1 was specific to the transendothelial migration assay, PBL and T cells were allowed to migrate to MCP-1 through 5 μm pore Transwells® with or without an endothelial monolayer. Both PBL and T cells were able to migrate to MCP-1 in the presence or absence of human endothelial cells (Fig. 16). Although the background migration was higher without the endothelial monolayer, it is apparent that the specific migration to MCP-1 is qualitatively similar in either the presence or absence of an endothelial monolayer.

To further understand the role of endothelial cells in the lymphocyte response to MCP-1, endothelial cells were incubated with MCP-1 (20 ng/ml) for 4 hr and their level of adhesion molecule expression was examined by flow cytometry. MCP-1 caused no significant upregulation in the expression of ICAM-1, ICAM-2, VCAM-1, E-selectin or P-selectin on the endothelial cells (data not shown). Additionally, supernatant from endothelial cells pretreated with MCP-1 was tested for lymphocyte chemotactic activity to rule out secondary endothelial cell effects. Specifically, endothelial cell monolayers were treated for 1 hr with 20 ng/ml MCP-1. The cells were washed to remove the MCP-1 and fresh media was added. After 4 hr, this conditioned media was harvested and tested. MCP-1-stimulated endothelial cell supernatant showed no activity above background in the lymphocyte transendothelial chemotaxis assay (data not shown). As a further control for secondary cell effects, monocytes were stimulated with MCP-1 as described above for the endothelial cell stimulation. After 4 hr this conditioned medium from MCP-1-stimulated monocytes was collected and tested for lymphocyte chemoattractant activity. No lymphocyte chemotactic activity was detected in this supernatant either (data not shown).

7.2.4. MONOCYTES MIGRATE TO MCP-1 WITH FASTER KINETICS THAN DO T CELLS

We compared the kinetics of lymphocyte and monocyte migration to MCP-1. PBMC subjected to 1 monocyte depletion were used in these

experiments to obtain a higher number of monocytes than in PBL preparations, which were subjected to 2 monocyte depletions. At early time points, $CD14^+$ monocytes predominated in the migrating population. The percentage of $CD3^+$ T lymphocytes that emigrated in response to MCP-1 increased with time, until they comprised 86% of the migrating cells at 8 hr. With PBL and purified T lymphocytes, T lymphocyte migration was significant at as early as 4 hr (data not shown).

7.2.5. PHENOTYPE OF LYMPHOCYTES MIGRATING TO MCP-1

The phenotype of lymphocytes in PBL preparations migrating to recombinant MCP-1 was determined by flow cytometric analysis of the starting population of cells as compared to cells that migrated into the lower chamber after 4 hr in the transendothelial chemotaxis assay (Fig. 17). In both cases, the cells were gated on the lymphocyte population based on their forward versus 90° scatter profile. Virtually no naive T cells, as defined by expression of CD45RA, responded to MCP-1. In contrast, memory cells appeared to be selected as shown by expression of CD45RO, increased expression of CD29, and the increased percentage of L-selectin$^-$ cells. The vast majority of migrating lymphocytes appeared to be activated, as demonstrated by their expression of the activation marker CD26. $CD4^+$ T cells seemed to preferentially migrate when compared to $CD8^+$ T cells. However the extent of this preferential response seems to vary from donor to donor. B cells and natural killer cells, as defined by the cell specific antigens CD20 and CD16, respectively, did not appear to migrate to MCP-1 at significant levels.

7.3. DISCUSSION

It has long been suspected that at sites of antigenic stimulation, a chemoattractant is secreted that is capable of recruiting lymphocytes from the bloodstream into the inflammatory lesion. Using the transendothelial lymphocyte chemotaxis assay, we have purified to homogeneity a lymphocyte chemoattractant in the supernatant of PHA-stimulated PBMC. Unexpectedly, amino acid sequence

analysis of this lymphocyte chemoattractant showed it to be identical to the previously characterized monocyte chemoattractant, MCP-1 (Yoshimura et al., 1989, J. Exp. Med. 169:1449; Matsushima et al., 1989, J. Exp. Med. 169:1485; Zachariae et al., 1990, J. Exp. Med. 171:2177). In several preparations, the majority of the material has a novelly processed N-terminus, beginning at position 3 compared to the previously reported MCP-1 sequence. A third form beginning at residue 6 has also been reported (Decock et al., 1990, Biochem. Biophys. Res. Commun. 167:904).

To confirm our results, we tested recombinant MCP-1 in our transendothelial chemotaxis assay. We showed that it attracts both purified T lymphocytes, as well as T lymphocytes within PBL preparations. Furthermore, we found that MCP-1 is responsible for the majority, but probably not all, of the T lymphocyte chemotactic activity within PHA supernatant, as shown by neutralization with antibody to MCP-1. We found that T cells respond to MCP-1 in a dose-dependent manner and by chemotaxis rather than chemokinesis, as demonstrated by checkerboard analysis.

The lymphocyte response to MCP-1 does not depend on the endothelium present in our assay system, since T lymphocytes migrate through filters to MCP-1 in the presence or absence of an endothelial cell monolayer. However, the endothelial cell monolayer greatly enhances the signal-to-noise ratio of the lymphocyte response to MCP-1 by decreasing non-specific migration to media controls. We ruled out secondary effects by showing that MCP-1 does not induce endothelial cells or monocytes to secrete a lymphocyte chemoattractant. Furthermore, although PHA supernatant potently induces expression of adhesion molecules including E-selectin, VCAM-1, and ICAM-1 on endothelium, the chemoattractant material purified past the gel filtration step, and recombinant MCP-1, do not. Thus, the migratory response to MCP-1 is not haptotactic.

Only a subpopulation of lymphocytes respond to MCP-1. The chemoattracted lymphocytes are all of the memory phenotype, as shown by the finding that they are $CD45RA^-$, $CD45RO^+$, $CD29^+$, and mostly $L\text{-selectin}^-$ (Mackay, 1992, Semin. Immunol. 4:51). Furthermore, they appear to be among

the most activated lymphocytes of the memory subset, as shown by increased expression of CD26, the ectoenzyme dipeptidyl peptidase IV expressed on activated T cells (Hegen et al., 1990, J. Immunol. 144:2908; Tanaka et al., 1993, Proc. Natl. Acad. Sci. USA 90:4586; Mattern et al., 1991, Scand. J. Immunol. 33:737). There seems to be a slight preference of $CD4^+$ cells over $CD8^+$ cells but the degree of this preference seems to vary between experiments. Additionally, neither $CD20^+$ B cells nor $CD16^+$ natural killer cells migrate to MCP-1 at significant levels.

In our transendothelial lymphocyte chemotaxis assay, we have found that the two α chemokines reported to induce T cell chemotaxis, IL-8 and IP-10, are inactive. The β chemokines, MIP-1α and RANTES, but not MIP-1β are active; however, MIP-1α and RANTES consistently attract fewer lymphocytes than does MCP-1.

The finding that MCP-1 acts as a T cell, as well as a monocyte chemoattractant, is a novel finding. Several groups have examined MCP-1 chemotactic specificity, yet none have reported a lymphocyte response (Matsushima et al., 1989, J. Exp. Med. 169:1485; Zachariae et al., 1990, J. Exp. Med. 171: 2177). In trying to understand why our findings differ from those of other groups, several possibilities come to mind. The first, and perhaps the most important, possibility is that because lymphocytes are less motile than neutrophils or monocytes, their chemotaxis has always been more difficult to measure (Parrott and Wilkinson, 1981, Prog. Allergy 28:193). Indeed, we found the kinetics of the lymphocyte and monocyte responses to MCP-1 to differ. Monocytes migrate significantly in as little as 2 hr while T lymphocyte migration is not significant until 4 hr. Many of the groups looking at migration to MCP-1 examined their chemotaxis assays at a timepoint of 90 min, and thus would have seen little lymphocyte migration (Yoshimura et al., 1989, J. Exp. Med. 169:1449, Matushima et al., 1989, J. Exp. Med. 169:1485; Yoshimura et al., 1989, J. Immunol. 142:1956). Because the endothelial barrier in our chemotaxis assay greatly slows the dissipation of the chemotactic gradient, chemotaxis can continue longer than in standard Boyden chamber assays where chemoattractants diffuse to

equilibrium within 4 hr (Lauffenburger and Zigmond, 1981, J. Immunol. Methods 40:45). Thus, the transendothelial variation of the traditional chemotaxis assay allows us to more easily detect the later-occurring T cell migration. Additionally, the presence of endothelium in our assay decreases non-specific background migration, allowing for small numbers of migrating T cells to be detected. The presence of the endothelial cell monolayer is not, however, required to observe T cell chemotaxis to MCP-1, as we have demonstrated. A second explanation is that much of the work with MCP-1 has been done looking at the migration of PBMC (Yoshimura et al., 1989, J. Exp. Med. 169:1449: Matsushima et al., 1989, J. Exp. Med. 169:1485; Yoshimura et al., 1989, J. Immunol. 142:1956), rather than with PBL or purified T lymphocytes as used here. We have found that with PBMC, which contain 20-30% monocytes, we were unable to detect T cell migration by flow cytometric analysis of the migrating cell population. We suspect that this is due to an overwhelming monocyte response which masks the response of T cells.

We have noticed variability in the overall magnitude of the T cell response to MCP-1. This variability may be specific to each T cell donor or it may be related to variations in the expression of undefined molecules on the surface of the endothelium which play a role in chemokine presentation. Experiments thus far have shown that cytokine activation of endothelium does not improve the chemotaxis assay, and in fact decreases the signal-to-background ratio.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for detecting or measuring lymphocyte chemotaxis comprising detecting or measuring the movement of lymphocytes through a filter, in a direction (a) toward increased levels of a known or suspected lymphocyte chemoattractant, and (b) from a first surface of the filter toward an opposite, second surface of the filter; wherein said filter is a microporous filter having on its first surface an endothelial cell monolayer.

2. A method for detecting or measuring lymphocyte chemotaxis comprising:

(a) providing an apparatus comprising:

(i) a microporous filter;

(ii) an endothelial cell monolayer on the upper surface of said filter;

(iii) a first chamber having an opening communicating with said endothelial cell monolayer, said first chamber containing a first composition, said first composition comprising a first fluid and lymphocytes; in which said first fluid contacts said endothelial cell monolayer; and

(iv) a second chamber having an opening communicating with the lower surface of said filter; in which said second chamber contains a second composition; said second composition (A) comprising a second fluid contacting the lower surface of said filter, and (B) comprising or suspected of comprising a molecule having lymphocyte chemoattractive activity;

(b) incubating the apparatus for a time period sufficient to allow any chemotaxis of lymphocytes to occur; and

(c) measuring the amount of lymphocytes in the second chamber, in which an increased amount of lymphocytes in the second

chamber relative to the baseline amount present when said second composition does not comprise a chemoattractant indicates that lymphocyte chemotaxis has occurred, and in which the difference between said increased amount and the baseline amount indicates the amount of lymphocyte chemotaxis.

3. The method according to claim 1 in which the microporous filter has a pore size in the range of 3-8 microns.

4. The method according to claim 1 in which the microporous filter has a pore size in the range of 5-8 microns.

5. The method according to claim 4 in which the endothelial cell is an umbilical vein endothelial cell.

6. The method according to claim 1 in which the lymphocytes are labelled.

7. The method according to claim 6 in which the label is fluorescent.

8. A method for detecting or measuring lymphocyte chemotaxis comprising detecting or measuring the movement of lymphocytes through a filter, in a direction (a) toward increased levels of a known or suspected lymphocyte chemoattractant, and (b) from a first surface of the filter toward an opposite, second surface of the filter; wherein said filter has a pore size in the range of 5-8 microns, and said filter has on its first surface a human umbilical vein endothelial cell monolayer.

9. A method for detecting or measuring lymphocyte chemotaxis comprising:

(a) providing an apparatus comprising:

(i) a filter having a pore size in the range of 5-8 microns;

(ii) a human umbilical vein endothelial cell monolayer on the upper surface of said filter;

(iii) a first chamber having an opening communicating with said endothelial cell monolayer; said first chamber containing a first composition, said first composition comprising a first fluid and lymphocytes, in which said first fluid contacts said endothelial cell monolayer; and

(iv) a second chamber having an opening communicating with the lower surface of the filter; in which said second chamber contains a second composition, said second composition comprising (A) a second fluid contacting the lower surface of the filter, and (B) a molecule having or suspected of having lymphocyte chemoattractive activity;

(b) incubating the apparatus for a time period sufficient to allow any chemotaxis of lymphocytes to occur; and

(c) measuring the amount of lymphocytes in the second chamber, in which an increased amount of lymphocytes in the second chamber relative to the baseline amount present when said second composition does not comprise a chemoattractant indicates that lymphocyte chemotaxis has occurred, and in which the difference between said increased amount and the baseline amount indicates the amount of lymphocyte chemotaxis.

10. The method according to claim 8 in which the lymphocytes are labeled.

11. The method according to claim 10 in which the lymphocytes are fluorescently labeled.

12. The method according to claim 11 in which the lymphocytes are labeled with fluorescein or a derivative thereof.

13. The method according to claim 2 in which the first composition has a cell population that is greater than 85% lymphocytes.

14. The method according to claim 9 in which the first composition has a cell population that is greater than 85% lymphocytes.

15. A method for identifying a lymphocyte chemoattractant comprising detecting lymphocyte chemotaxis according to the method of claim 1, in which said movement is toward increased levels of a suspected chemoattractant, and in which the detection of said movement indicates that the suspected chemoattractant is a chemoattractant.

16. A method for identifying a lymphocyte chemoattractant comprising detecting lymphocyte chemotaxis according to the method of claim 8, in which said movement is toward increased levels of a suspected chemoattractant, and in which the detection of said movement indicates that the suspected chemoattractant is a chemoattractant.

17. A method for identifying a lymphocyte chemoattractant comprising detecting lymphocyte chemotaxis according to the method of claim 2, in which the detection of lymphocyte chemotaxis indicates that said molecule present in the second composition is a lymphocyte chemoattractant.

18. A method for identifying a lymphocyte chemoattractant comprising detecting lymphocyte chemotaxis according to the method of claim 9, in which the detection of lymphocyte chemotaxis indicates that said molecule comprised in the second composition is a lymphocyte chemoattractant.

19. A method for identifying a lymphocyte chemoattractant comprising detecting lymphocyte chemotaxis according to the method of claim 13, in which the detection of lymphocyte chemotaxis indicates that said molecule comprised in the second composition is a lymphocyte chemoattractant.

20. A method for identifying a lymphocyte chemoattractant comprising detecting lymphocyte chemotaxis according to the method of claim 14, in which the detection of lymphocyte chemotaxis indicates that said molecule comprised in the second composition is a lymphocyte chemoattractant.

21. A purified molecule characterized by the following properties:

(a) chemoattractive activity for T lymphocytes, as detected in the assay of claim 8;

(b) an amino-terminal sequence as follows: Asp-Ala-Ile-Asn-Ala-Pro-Val; and

(c) a molecular weight of about 16,000 daltons, as measured by size exclusion chromatography.

22. A purified molecule having the amino acid sequence depicted in Figure 18 from amino acid numbers 3-76.

23. A pharmaceutical composition comprising a pharmaceutically acceptable carrier; and a therapeutically effective amount of the molecule of claim 21.

24. A pharmaceutical composition comprising a pharmaceutically acceptable carrier; and a therapeutically effective amount of the molecule of claim 22.

25. A method for detecting a lymphocyte chemoattractant antagonist comprising:

(a) providing an apparatus comprising:

(i) a microporous filter;

(ii) an endothelial cell monolayer on the upper surface of said filter;

(iii) a first chamber having an opening communicating with said endothelial cell monolayer, said first chamber containing a first composition, said first composition comprising (A) a first fluid contacting said endothelial cell monolayer, (B) a test molecule, and (C) lymphocytes; and

(iv) a second chamber having an opening communicating with the lower surface of said filter, in which said second chamber contains a second composition; said second composition comprising (A) a second fluid contacting the lower surface of said filter, and (B) a lymphocyte chemoattractant;

(b) incubating the apparatus for a time period sufficient to allow any chemotaxis of lymphocytes to occur; and

(c) measuring the amount of lymphocytes in the second chamber, in which a decreased amount of lymphocytes in the second chamber relative to the amount of lymphocytes in the second chamber measured when the first composition does not contain the test molecule, indicates that the test molecule is a lymphocyte chemoattractant antagonist.

26. A substantially purified lymphocyte chemoattractant antagonist which is detected according to the method of claim 25.

27. A pharmaceutical composition comprising the lymphocyte chemoattractant antagonist of claim 26.

28. A method of treating a disease or disorder associated with an undesirable inflammatory response in a patient comprising administering into the bloodstream of the patient a therapeutically effective amount of the molecule of claim 21.

29. A method of treating a disease or disorder associated with an undesirable inflammatory response in a patient comprising administering into the bloodstream of the patient a therapeutically effective amount of the molecule of claim 22.

30. A method of treating a tumor in a patient comprising locally delivering to the tumor a therapeutically effective amount of the molecule of claim 21.

31. A method of treating a tumor in a patient comprising locally delivering to the tumor a therapeutically effective amount of the molecule of claim 22.

32. The method according to claim 30 in which the local delivery is by direct injection into the tumor.

33. A composition comprising the molecule of claim 21 for use in a method of treatment.

34. A composition comprising the molecule of claim 22 for use in a method of treatment.

35. A method for making an artificial vessel construct comprising growing an endothelial cell monolayer on a surface of a filter, said filter having a pore size in the range of 4-7 microns.

36. The method according to claim 35 in which the pore size is 5 microns.

37. An artificial vessel construct comprising:

(a) a filter having a pore size in the range of 4-7 microns; and

(b) an endothelial cell monolayer on a surface of said filter.

38. The artificial vessel construct of claim 37 in which the pore size is 5 microns.

39. An apparatus for detecting or measuring lymphocyte chemotaxis comprising:

(a) a filter having a pore size in the range of 4-7 microns;

(b) an endothelial cell monolayer on a first surface of said filter;

(c) a first chamber having an opening communicating with said endothelial cell monolayer; and

(d) a second chamber having an opening communicating with a second, opposite surface of the filter.

40. The apparatus of claim 39 in which the pore size is 5 microns.

41. A method of diagnosing an inflammatory disorder in a patient comprising measuring lymphocyte chemotaxis according to the method of claim 1, in which said lymphocytes are from the patient; and said movement is toward a known lymphocyte chemoattractant; in which a decrease in the measured amount of lymphocyte chemotaxis relative to the level observed with lymphocytes from a healthy individual or from the patient prior to disease onset or in remission, indicates the presence of an inflammatory disorder.

42. The method according to claim 2 in which the amount of lymphocytes in the second chamber measured in step (d) is calculated as the ratio

of the number of cells present in the second chamber to the number of cells in said first composition.

AMENDED CLAIMS

[received by the International Bureau on 11 July 1994 (11.07.94); original claims 35-38 cancelled; original claims 1,2,8,9,15-20, 25,39,41 and 42 amended; remaining claims unchanged; claims renumbered 1-38 (9 pages)]

1. A method for detecting or measuring lymphocyte chemotaxis comprising detecting or measuring the transmigration of lymphocytes through a filter, in a direction (a) toward increased levels of a known or suspected lymphocyte chemoattractant, and (b) from a first surface of the filter toward an opposite, second surface of the filter; wherein said filter is a microporous filter having on its first surface an endothelial cell monolayer; and wherein the presence and amount of said transmigration of lymphocytes through the filter indicates the presence and amount, respectively, of lymphocyte chemotaxis.

2. A method for detecting or measuring lymphocyte chemotaxis comprising:

(a) incubating an apparatus for a time period sufficient to allow any chemotaxis of lymphocytes to occur, in which said apparatus comprises:

(i) a microporous filter;

(ii) an endothelial cell monolayer on an upper surface of said filter;

(iii) a first chamber having an opening communicating with said endothelial cell monolayer, said first chamber containing a first composition, said first composition comprising a first fluid and lymphocytes; in which said first fluid contacts said endothelial cell monolayer; and

(iv) a second chamber having an opening communicating with a lower surface of said filter; in which said second chamber contains a second composition; said second composition (A) comprising a second fluid contacting the lower surface of said filter, and (B) comprising or suspected of comprising molecules having lymphocyte chemoattractive activity; and

(b) measuring the amount of lymphocytes in the second chamber, in which an increased amount of lymphocytes in the second chamber relative to the baseline amount present when said second composition does not comprise a lymphocyte chemoattractant indicates that lymphocyte chemotaxis has occurred, and in which the difference between said increased amount and the baseline amount indicates the amount of lymphocyte chemotaxis.

3. The method according to claim 1 in which the microporous filter has a pore size in the range of 3-8 microns.

4. The method according to claim 1 in which the microporous filter has a pore size in the range of 5-8 microns.

5. The method according to claim 4 in which the endothelial cell is an umbilical vein endothelial cell.

6. The method according to claim 1 in which the lymphocytes are labelled.

7. The method according to claim 6 in which the label is fluorescent.

8. A method for detecting or measuring lymphocyte chemotaxis comprising detecting or measuring the transmigration of lymphocytes through a filter, in a direction (a) toward increased levels of a known or suspected lymphocyte chemoattractant, and (b) from a first surface of the filter toward an opposite, second surface of the filter; wherein said filter has a pore size in the range of 5-8 microns, and said filter has on its first surface a human umbilical vein endothelial cell monolayer; and wherein the presence and amount of said

transmigration of lymphocytes through the filter indicates the presence and amount respectively, of lymphocyte chemotaxis.

9. A method for detecting or measuring lymphocyte chemotaxis comprising:

(a) incubating an apparatus for a time period sufficient to allow any chemotaxis of lymphocytes to occur, in which said apparatus comprises:

(i) a filter having a pore size in the range of 5-8 microns;

(ii) a human umbilical vein endothelial cell monolayer on an upper surface of said filter;

(iii) a first chamber having an opening communicating with said endothelial cell monolayer; said first chamber containing a first composition, said first composition comprising a first fluid and lymphocytes, in which said first fluid contacts said endothelial cell monolayer; and

(iv) a second chamber having an opening communicating with a lower surface of the filter; in which said second chamber contains a second composition, said second composition comprising (A) a second fluid contacting the lower surface of the filter, and (B) molecules having or suspected of having lymphocyte chemoattractive activity; and

(b) measuring the amount of lymphocytes in the second chamber, in which an increased amount of lymphocytes in the second chamber relative to the baseline amount present when said second composition does not comprise a lymphocyte chemoattractant indicates that lymphocyte chemotaxis has occurred, and in which the difference between said increased amount and the baseline amount indicates the amount of lymphocyte chemotaxis.

10. The method according to claim 8 in which the lymphocytes are labeled.

11. The method according to claim 10 in which the lymphocytes are fluorescently labeled.

12. The method according to claim 11 in which the lymphocytes are labeled with fluorescein or a derivative thereof.

13. The method according to claim 2 in which the first composition has a cell population that is greater than 85% lymphocytes.

14. The method according to claim 9 in which the first composition has a cell population that is greater than 85% lymphocytes.

15. A method for identifying a lymphocyte chemoattractant comprising detecting lymphocyte chemotaxis according to the method of claim 1, in which said transmigration is toward increased levels of a suspected lymphocyte chemoattractant, and in which the detection of said transmigration indicates that the suspected lymphocyte chemoattractant is a lymphocyte chemoattractant.

16. A method for identifying a lymphocyte chemoattractant comprising detecting lymphocyte chemotaxis according to the method of claim 8, in which said transmigration is toward increased levels of a suspected lymphocyte chemoattractant, and in which the detection of said transmigration indicates that the suspected lymphocyte chemoattractant is a lymphocyte chemoattractant.

17. A method for identifying one or more lymphocyte chemoattractants comprising detecting lymphocyte chemotaxis according to the method of claim 2, in which the detection of lymphocyte chemotaxis indicates that said molecules comprised in the second composition are lymphocyte chemoattractants.

18. A method for identifying one or more lymphocyte chemoattractants comprising detecting lymphocyte chemotaxis according to the method of claim 9, in which the detection of lymphocyte chemotaxis indicates that said molecules comprised in the second composition are lymphocyte chemoattractants.

19. A method for identifying one or more lymphocyte chemoattractants comprising detecting lymphocyte chemotaxis according to the method of claim 13, in which the detection of lymphocyte chemotaxis indicates that said molecules comprised in the second composition are lymphocyte chemoattractants.

20. A method for identifying one or more lymphocyte chemoattractants comprising detecting lymphocyte chemotaxis according to the method of claim 14, in which the detection of lymphocyte chemotaxis indicates that said molecules comprised in the second composition are lymphocyte chemoattractants.

21. A purified molecule characterized by the following properties:

(a) chemoattractive activity for T lymphocytes, as detected in the assay of claim 8;

(b) an amino-terminal sequence as follows:
Asp-Ala-Ile-Asn-Ala-Pro-Val; and

(c) a molecular weight of about 16,000 daltons, as measured by size exclusion chromatography.

22. A purified molecule having the amino acid sequence depicted in Figure 18 from amino acid numbers 3-76.

23. A pharmaceutical composition comprising a pharmaceutically acceptable carrier; and a therapeutically effective amount of the molecule of claim 21.

24. A pharmaceutical composition comprising a pharmaceutically acceptable carrier; and a therapeutically effective amount of the molecule of claim 22.

25. A method for detecting a lymphocyte chemoattractant antagonist comprising:

(a) incubating an apparatus for a time period sufficient to allow any chemotaxis of lymphocytes to occur, in which said apparatus comprises:

(i) a microporous filter;

(ii) an endothelial cell monolayer on an upper surface of said filter;

(iii) a first chamber having an opening communicating with said endothelial cell monolayer, said first chamber containing a first composition, said first composition comprising (A) a first fluid contacting said endothelial cell monolayer, (B) test molecules, and (C) lymphocytes; and

(iv) a second chamber having an opening communicating with a lower surface of said filter, in which said second chamber contains a second composition; said second composition comprising (A) a second fluid contacting the lower surface of said filter, and (B) lymphocyte chemoattractant molecules; and

(b) measuring the amount of lymphocytes in the second chamber, in which a decreased amount of lymphocytes in the second chamber relative to the amount of lymphocytes in the second chamber measured when the first composition does not contain the test molecules, indicates that the test molecules are lymphocyte chemoattractant antagonists.

26. A substantially purified lymphocyte chemoattractant antagonist which is detected according to the method of claim 25.

27. A pharmaceutical composition comprising the lymphocyte chemoattractant antagonist of claim 26.

28. A method of treating a disease or disorder associated with an undesirable inflammatory response in a patient comprising administering into the bloodstream of the patient a therapeutically effective amount of the molecule of claim 21.

29. A method of treating a disease or disorder associated with an undesirable inflammatory response in a patient comprising administering into the bloodstream of the patient a therapeutically effective amount of the molecule of claim 22.

30. A method of treating a tumor in a patient comprising locally delivering to the tumor a therapeutically effective amount of the molecule of claim 21.

31. A method of treating a tumor in a patient comprising locally delivering to the tumor a therapeutically effective amount of the molecule of claim 22.

32. The method according to claim 30 in which the local delivery is by direct injection into the tumor.

33. A composition comprising the molecule of claim 21 for use in a method of treatment.

34. A composition comprising the molecule of claim 22 for use in a method of treatment.

35. An apparatus for detecting or measuring lymphocyte chemotaxis comprising:

(a) a filter having a pore size in the range of 4-7 microns;

(b) an endothelial cell monolayer on a first surface of said filter;

(c) a first chamber having an opening communicating with said endothelial cell monolayer; and

(d) a second chamber having an opening communicating with a second, opposite surface of the filter.

in which said filter having said endothelial cell monolayer on its first surface allows the transmigration of lymphocytes, when present in said first chamber, through the filter into said second chamber when said second chamber contains lymphocyte chemoattractant molecules at an increased level relative to the level of lymphocyte chemoattractant molecules in said first chamber, when said apparatus is incubated for a time period in the range of three to six hours.

36. The apparatus of claim 35 in which the pore size is 5 microns.

37. A method of diagnosing an inflammatory disorder in a patient comprising measuring lymphocyte chemotaxis according to the method of claim 1, in which said lymphocytes are from the patient; and said transmigration is toward known lymphocyte chemoattractant molecules; in which a decrease in the measured amount of lymphocyte chemotaxis relative to the level observed with lymphocytes from a healthy individual or from the patient prior to disease onset or in remission, indicates the presence of an inflammatory disorder.

38. The method according to claim 2 in which the amount of lymphocytes in the second chamber measured in step (d) is calculated as the ratio

of the number of lymphocytes present in the second chamber to the number of lymphocytes in said first composition.

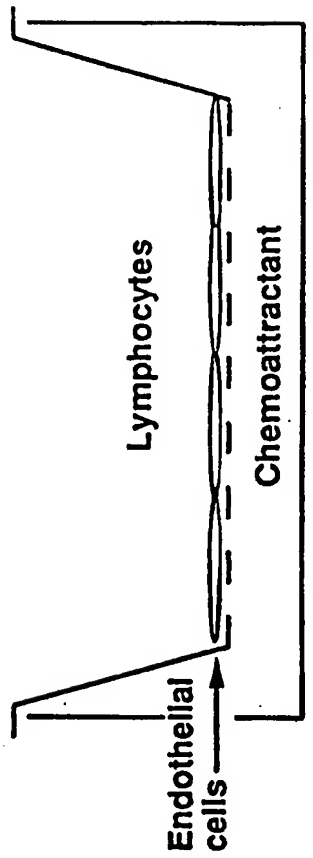
Lymphocytes
Chemoattractant
Endothelial cells

FIG. 1B

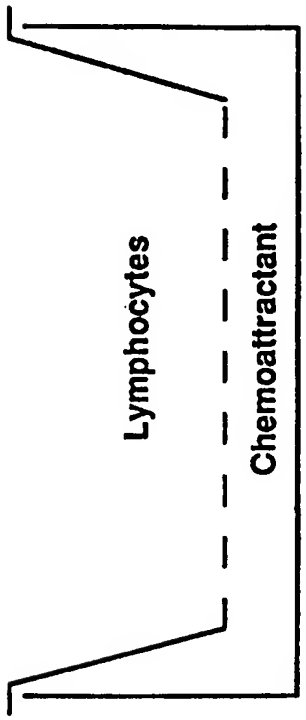
Lymphocytes
Chemoattractant

FIG. 1A

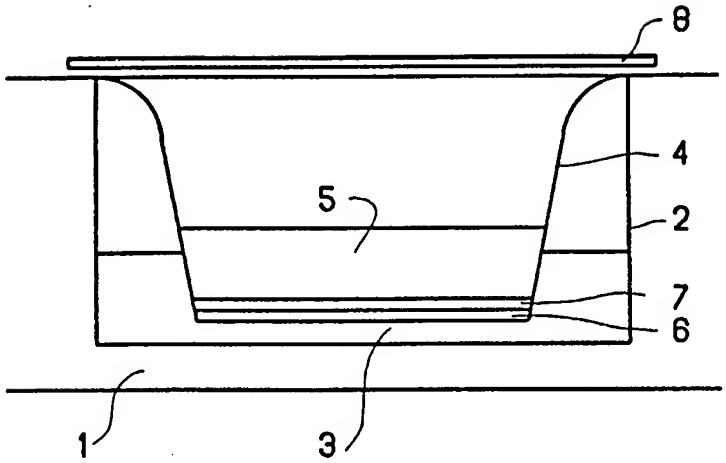

FIG. 2

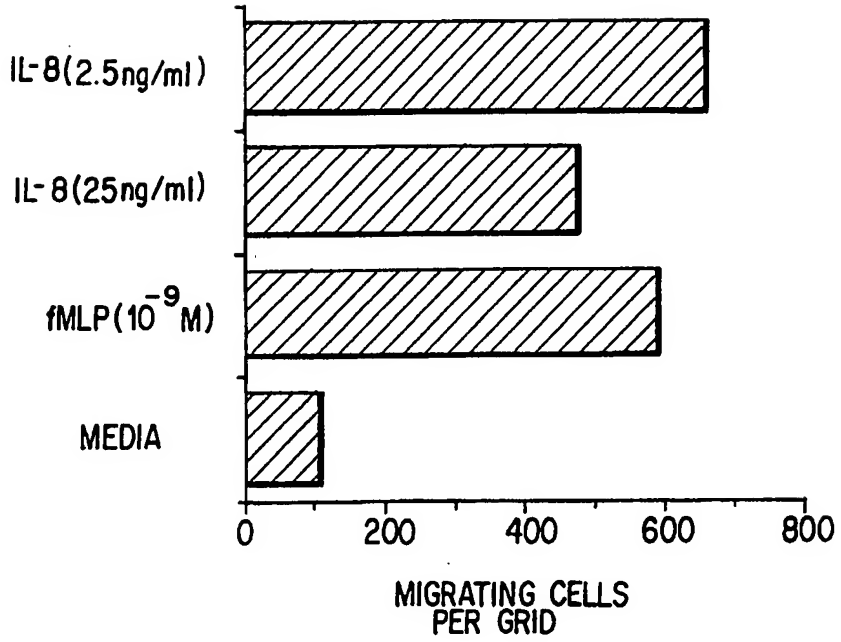
IL-8(2.5ng/ml)
IL-8(25ng/ml)
fMLP(10^{-9}M)
MEDIA
0
200
400
600
800
MIGRATING CELLS PER GRID

FIG. 3A

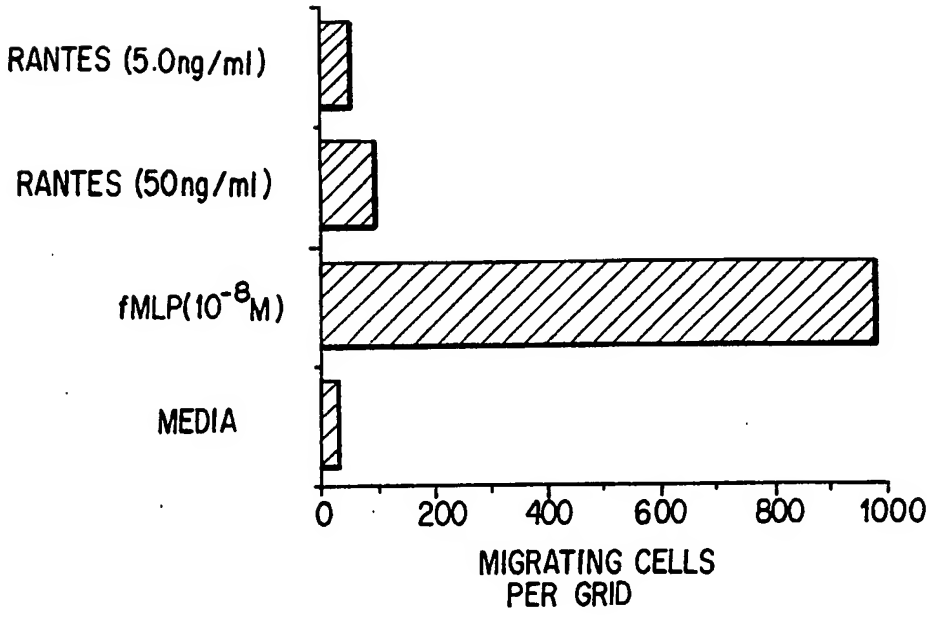
RANTES (5.0ng/ml)
RANTES (50ng/ml)
fMLP(10^{-8}M)
MEDIA
0
200
400
600
800
1000
MIGRATING CELLS PER GRID

FIG. 3B

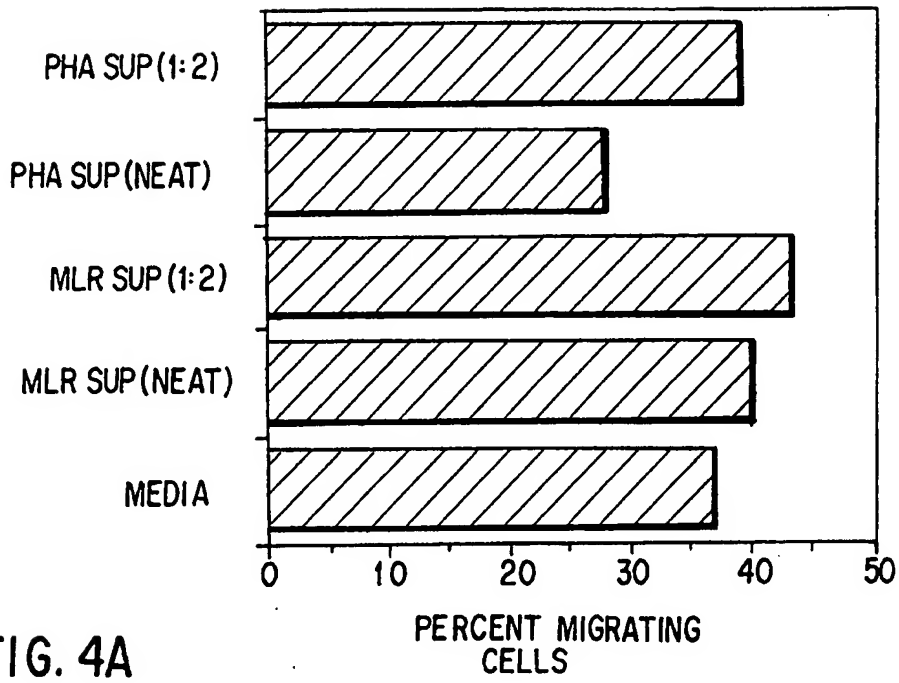
PHA SUP(1:2)
PHA SUP(NEAT)
MLR SUP(1:2)
MLR SUP(NEAT)
MEDIA
0
10
20
30
40
50
PERCENT MIGRATING CELLS

FIG. 4A

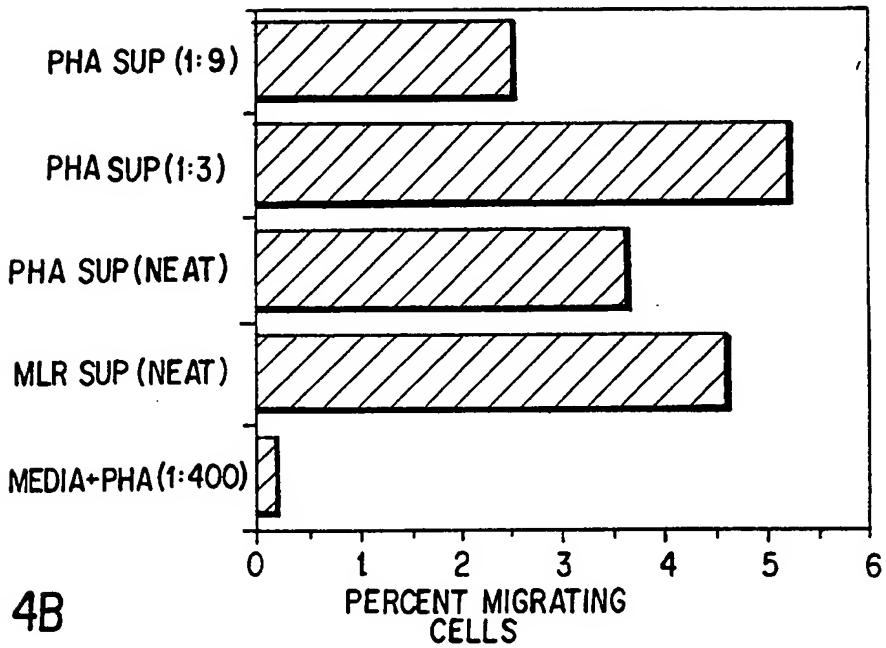
PHA SUP (1:9)
PHA SUP(1:3)
PHA SUP(NEAT)
MLR SUP (NEAT)
MEDIA+PHA(1:400)
0
1
2
3
4
5
6
PERCENT MIGRATING CELLS

FIG. 4B

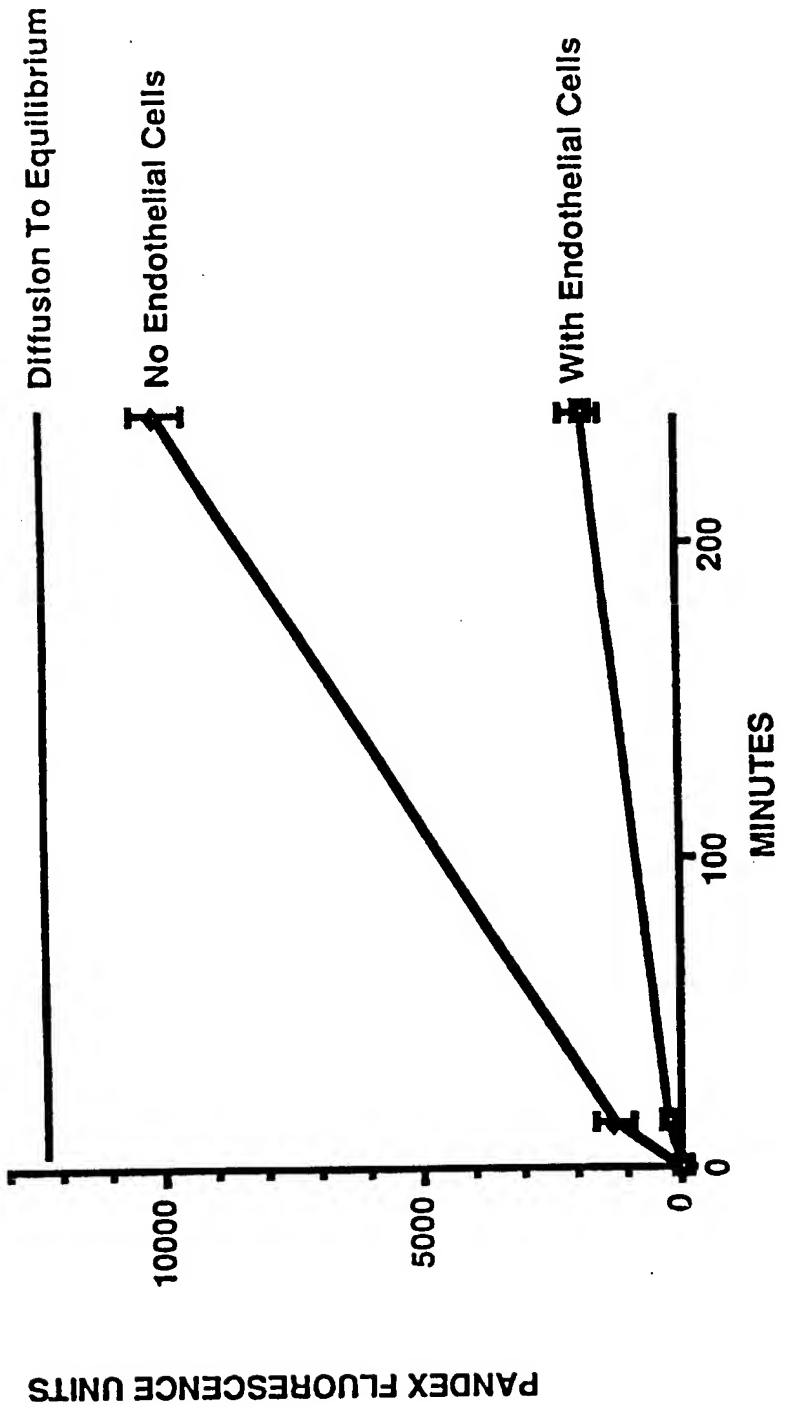
Diffusion To Equilibrium
No Endothelial Cells
With Endothelial Cells
0
100
200
MINUTES
0
5000
10000
PANDEX FLUORESCENCE UNITS

FIG. 5

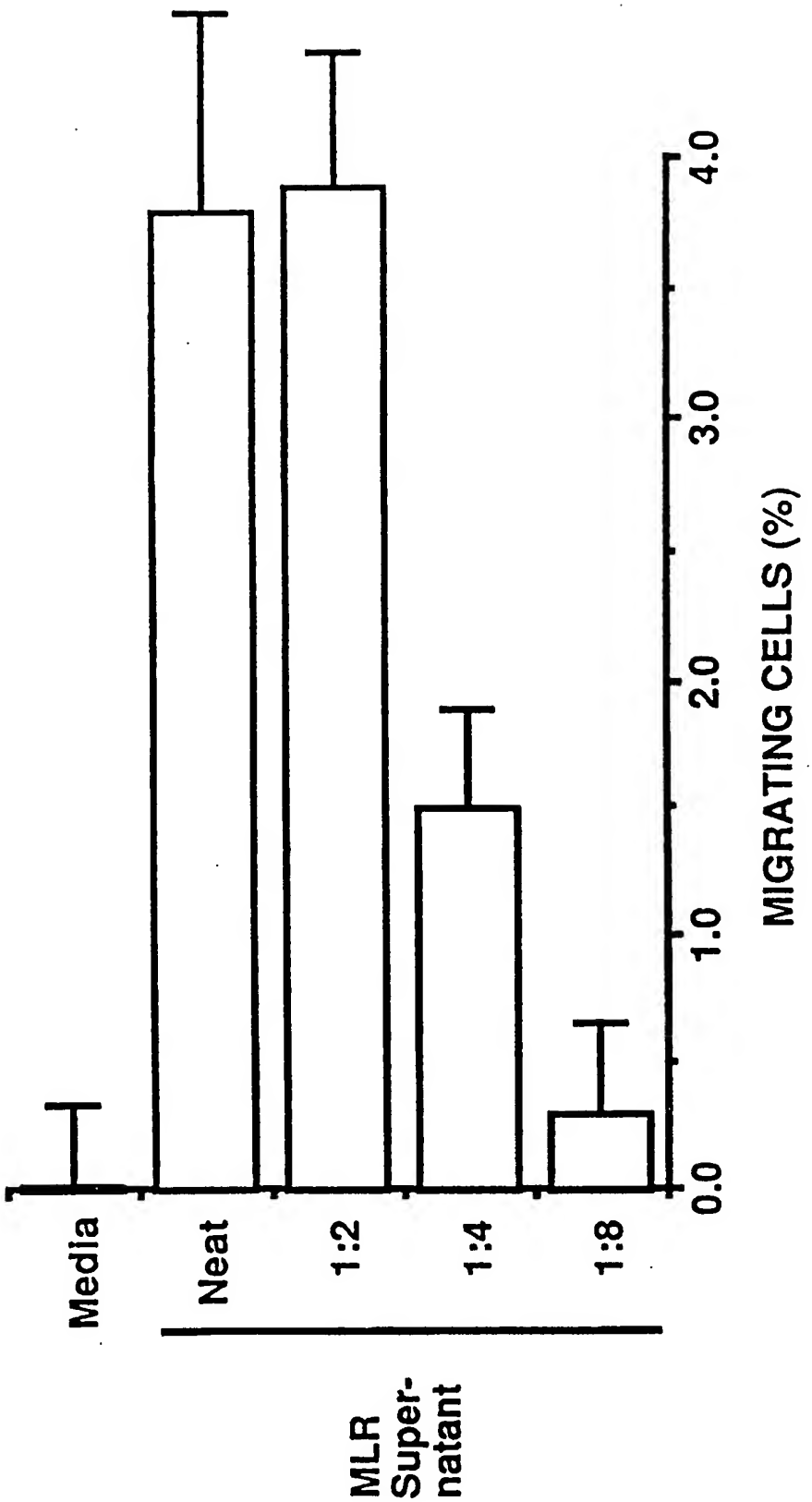
MIGRATING CELLS (%)
0.0
1.0
2.0
3.0
4.0
Media
Neat
1:2
1:4
1:8
MLR Super-natant

FIG.6

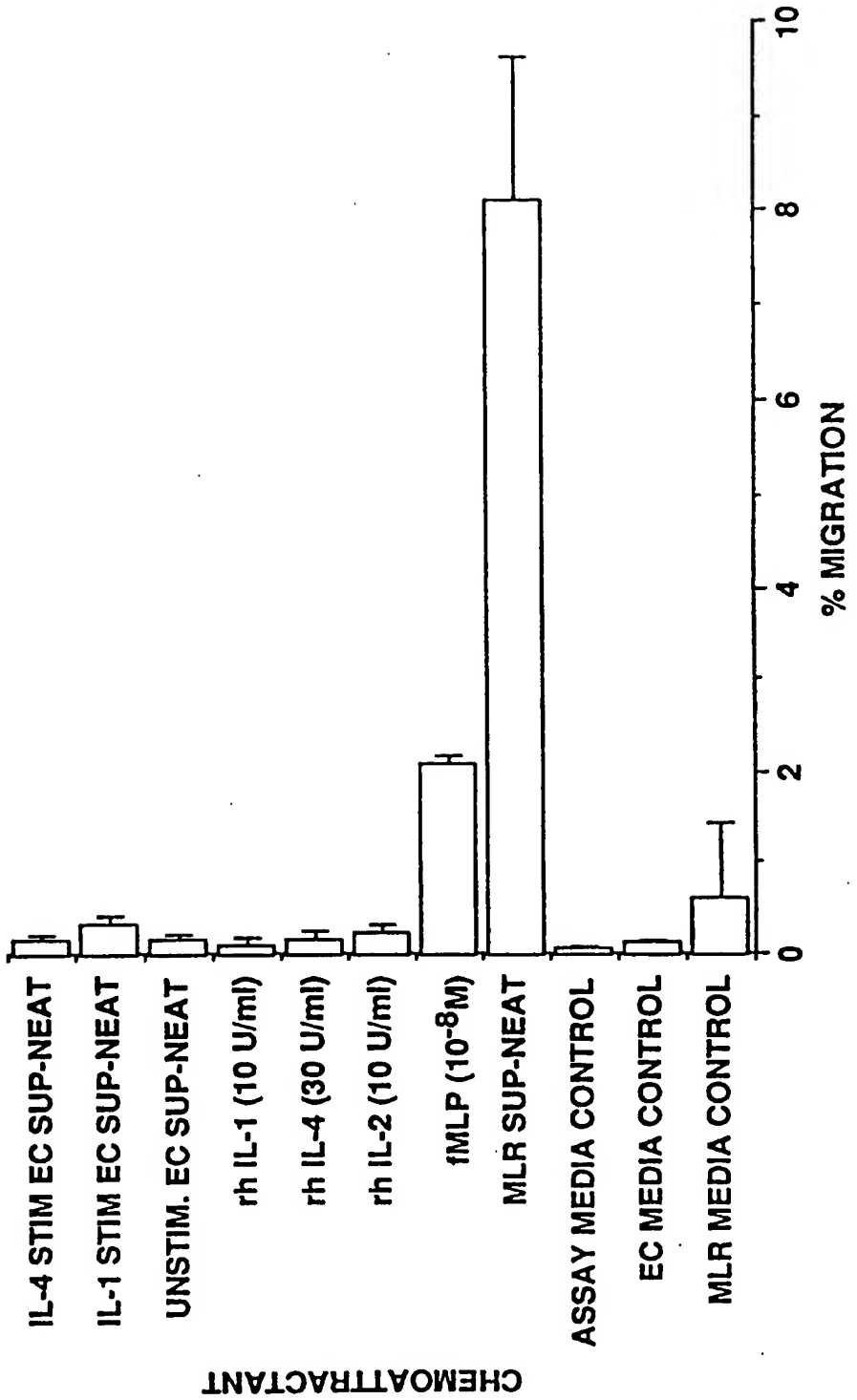
% MIGRATION
0
2
4
6
8
10
IL-4 STIM EC SUP-NEAT
IL-1 STIM EC SUP-NEAT
UNSTIM. EC SUP-NEAT
rh IL-1 (10 U/ml)
rh IL-4 (30 U/ml)
rh IL-2 (10 U/ml)
fMLP (10^{-8}M)
MLR SUP-NEAT
ASSAY MEDIA CONTROL
EC MEDIA CONTROL
MLR MEDIA CONTROL
CHEMOATTRACTANT

FIG. 7

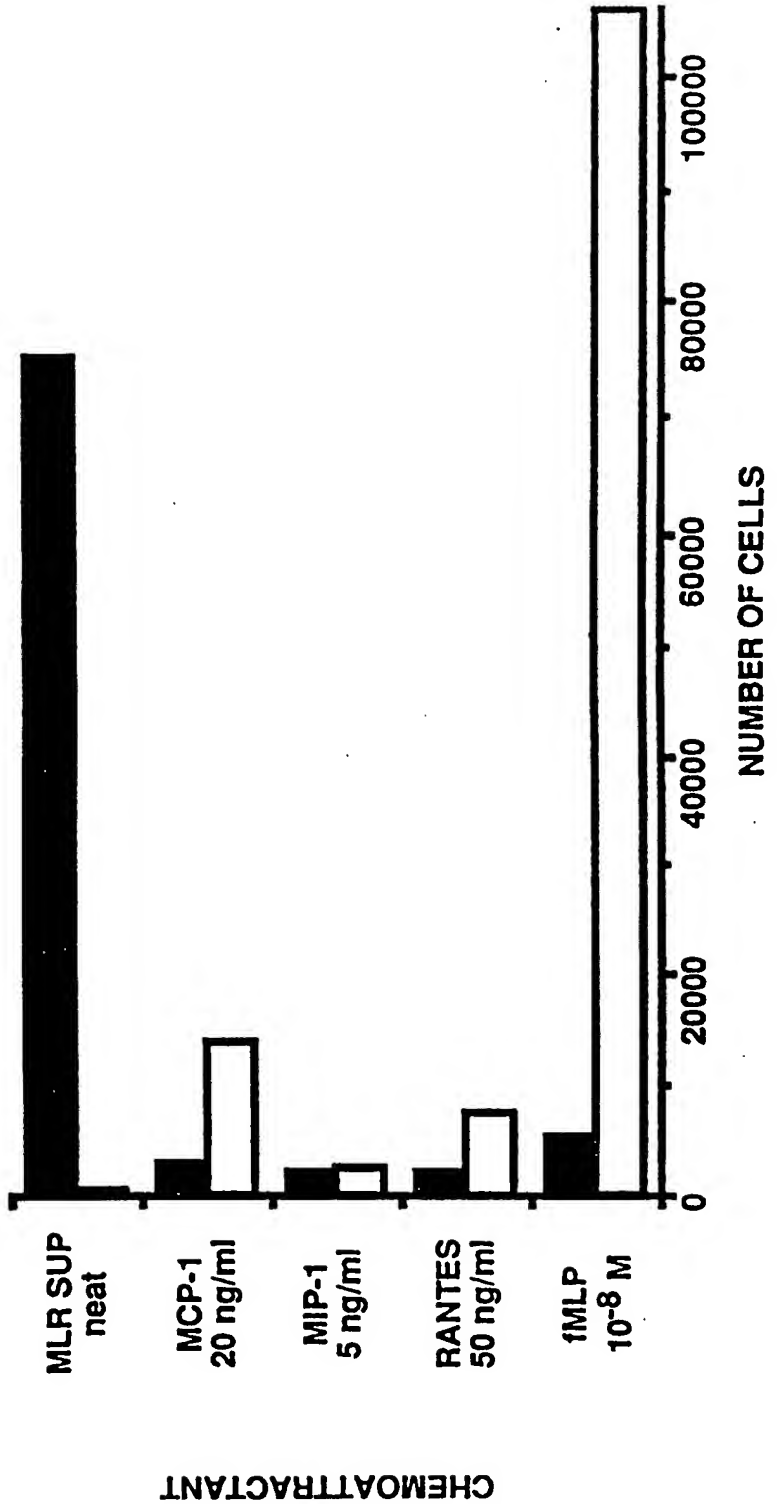
NUMBER OF CELLS
0
20000
40000
60000
80000
100000
MLR SUP neat
MCP-1 20 ng/ml
MIP-1 5 ng/ml
RANTES 50 ng/ml
fMLP 10^{-8} M
CHEMOATTRACTANT

FIG. 8

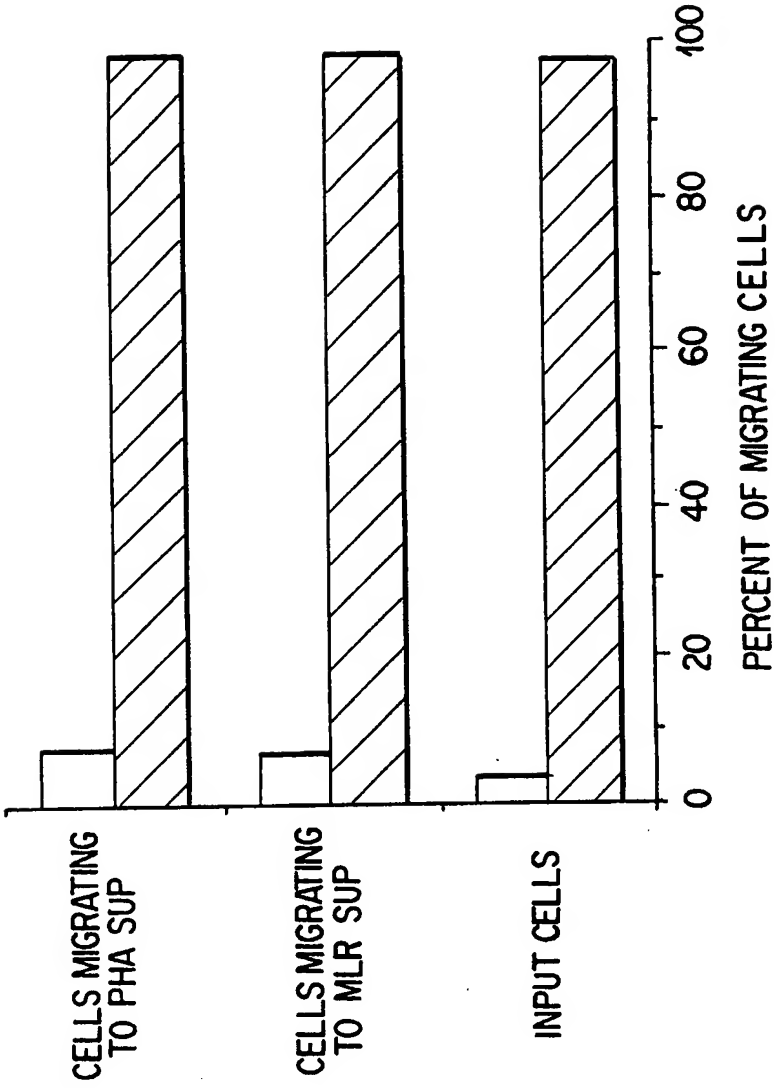
CELLS MIGRATING TO PHA SUP
CELLS MIGRATING TO MLR SUP
INPUT CELLS
0
20
40
60
80
100
PERCENT OF MIGRATING CELLS

FIG.9

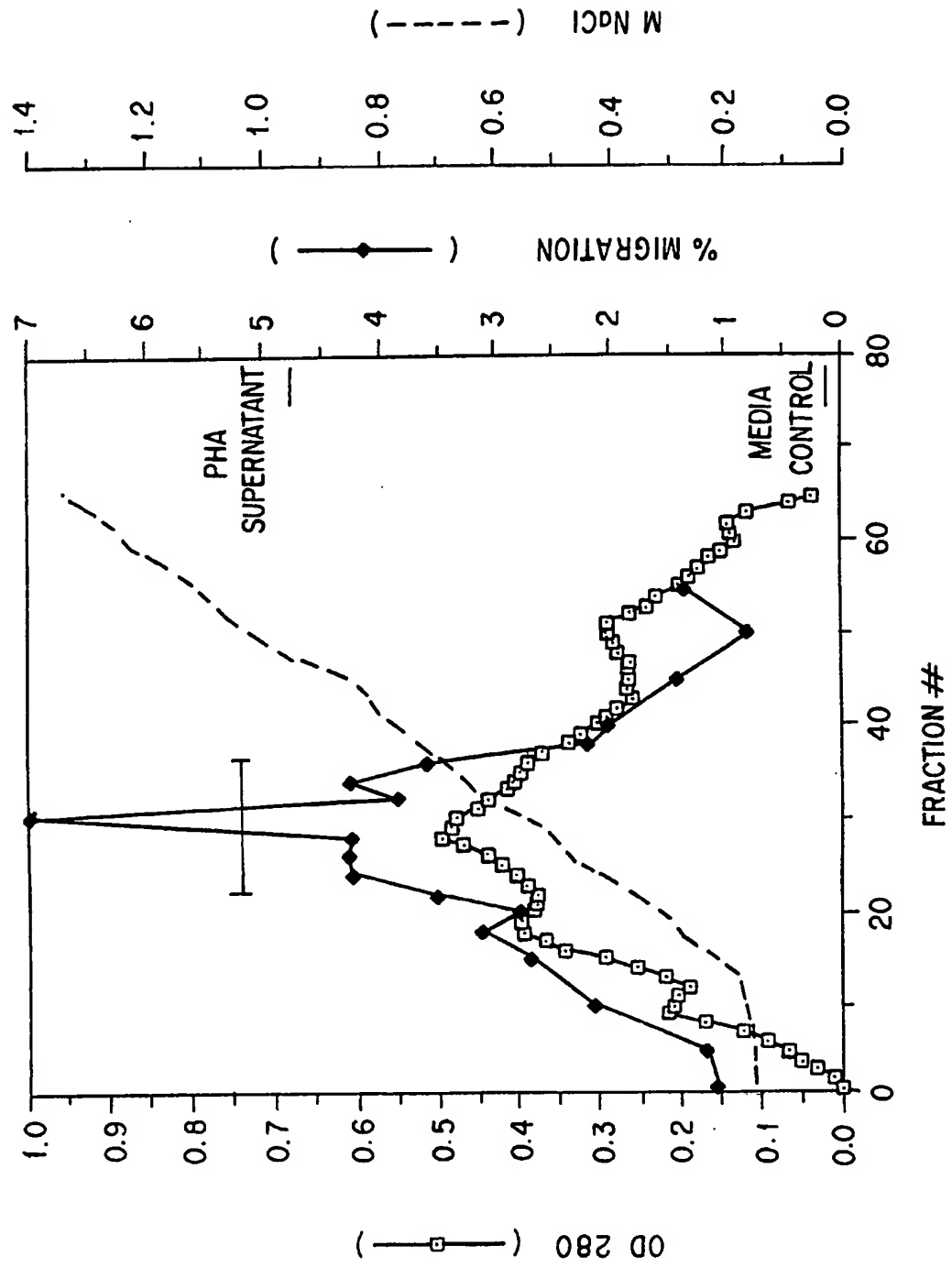
M NaCl (- - - - -)
% MIGRATION (—◆—)
OD 280 (—□—)
PHA SUPERNATANT
MEDIA CONTROL
FRACTION #

FIG. 10

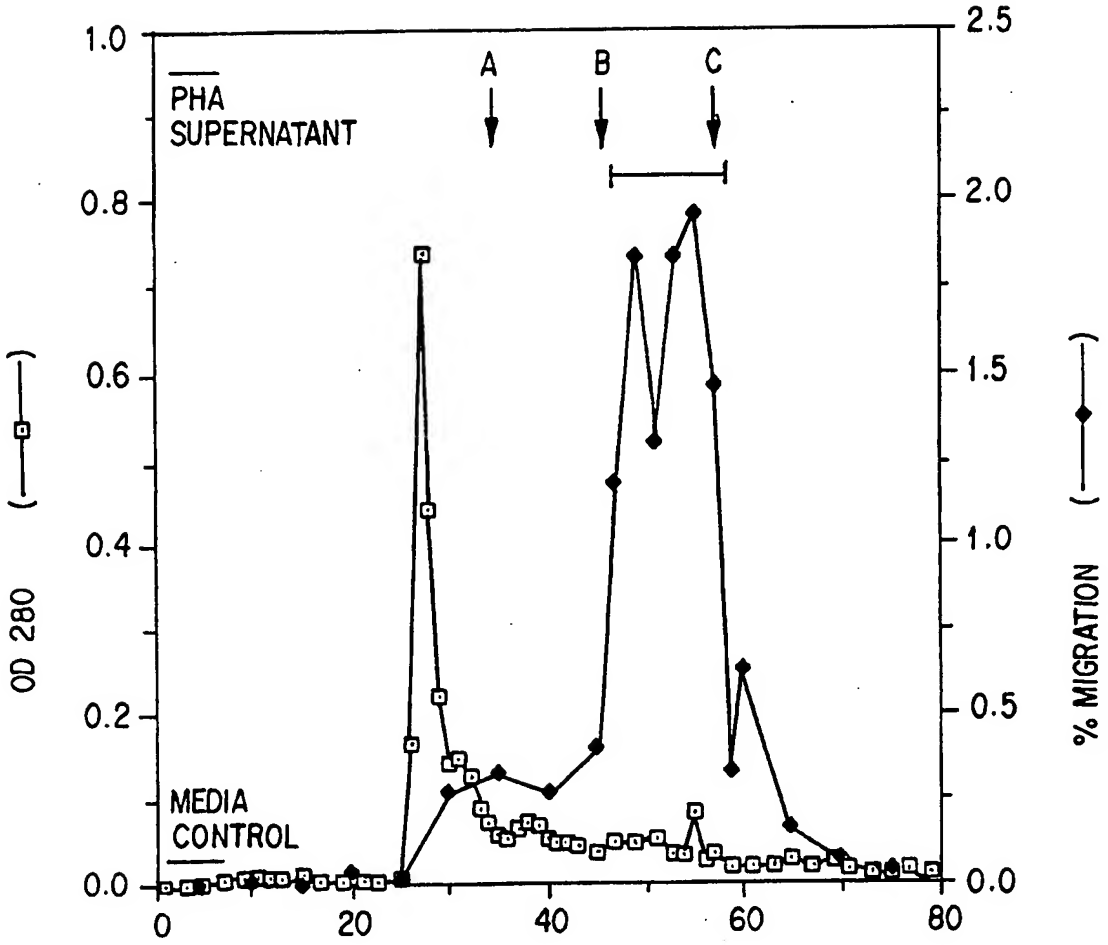
1.0
0.8
0.6
0.4
0.2
0.0
OD 280
2.5
2.0
1.5
1.0
0.5
0.0
% MIGRATION
A
B
C
PHA
SUPERNATANT
MEDIA
CONTROL
0
20
40
60
80
FRACTION #

FIG. 11

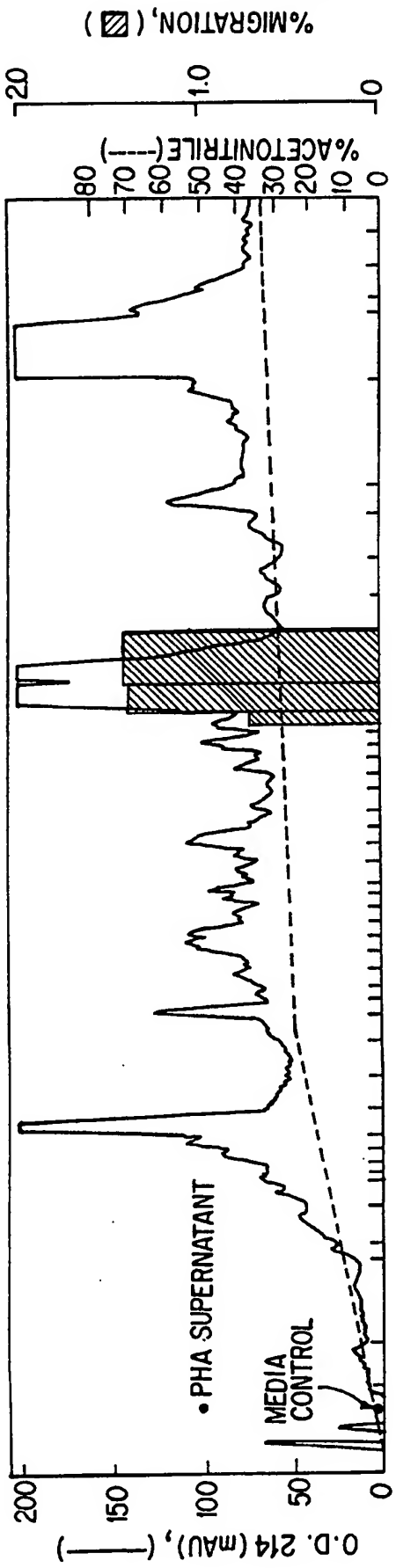
%MIGRATION, (▨)
20
1.0
0
%ACETONITRILE (-----)
80
70
60
50
40
30
20
10
0
• PHA SUPERNATANT
MEDIA CONTROL
200
150
100
50
0
O.D. 214 (mAU), (——)

FIG.12

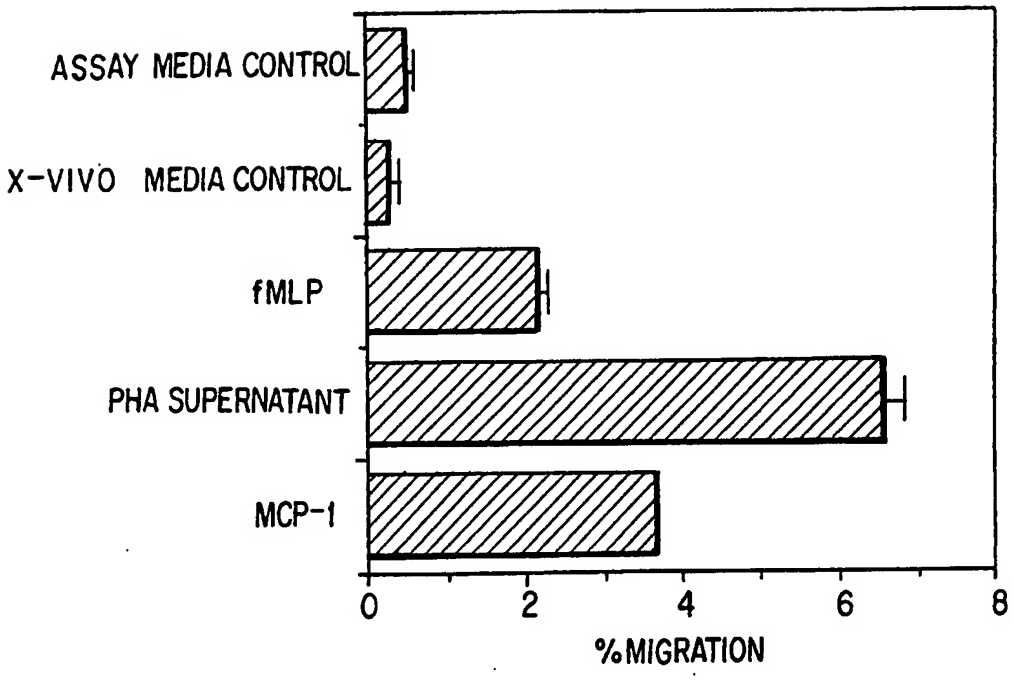

FIG. 13A

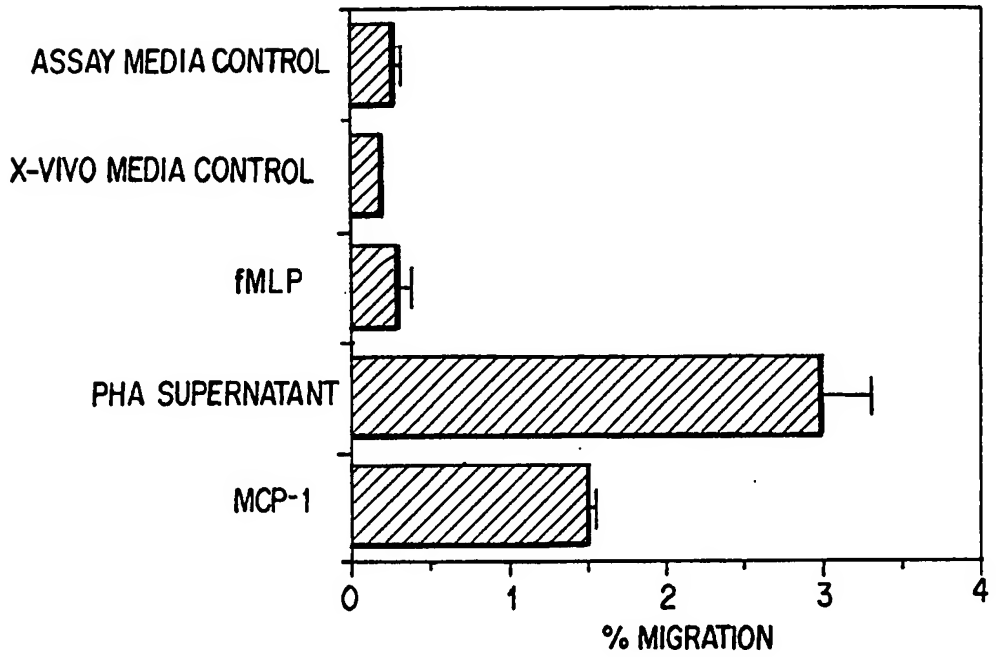

FIG. 13B

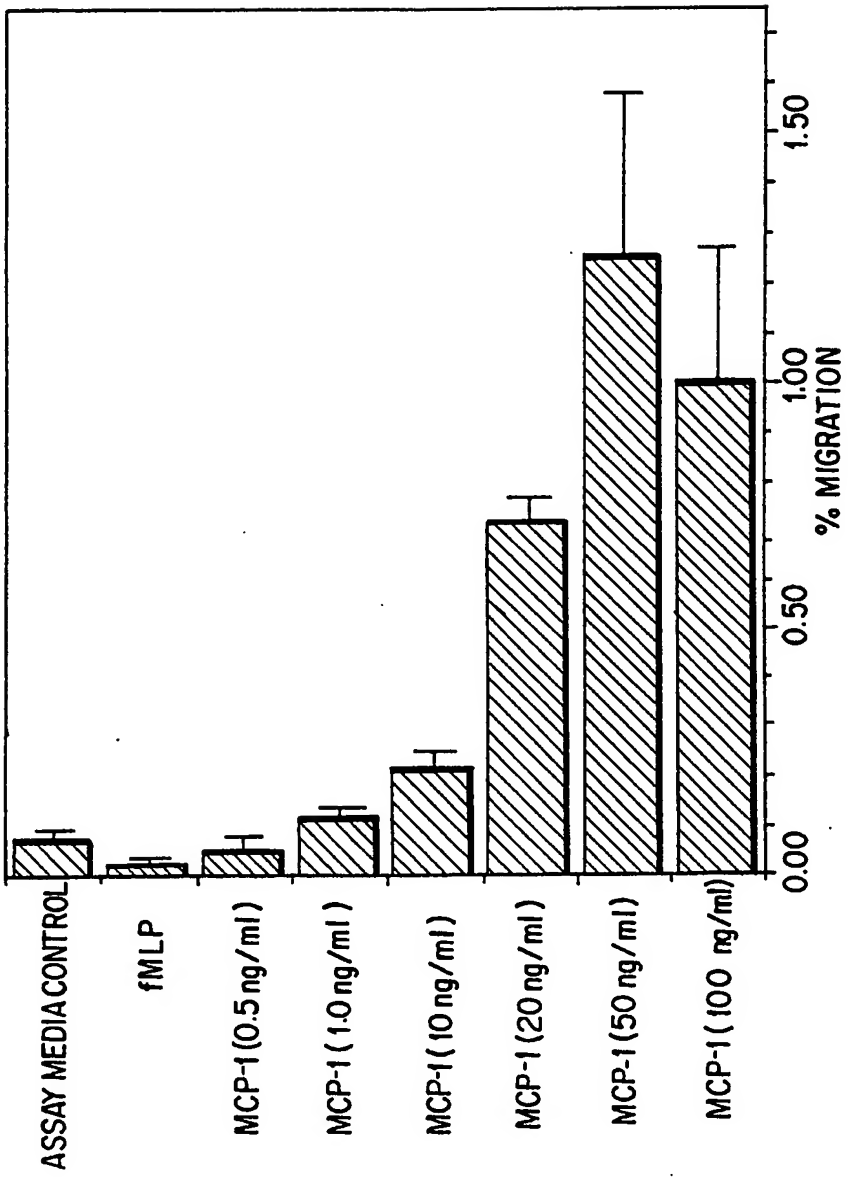
0.00
0.50
1.00
1.50
% MIGRATION
ASSAY MEDIA CONTROL
fMLP
MCP-1 (0.5 ng/ml)
MCP-1 (1.0 ng/ml)
MCP-1 (10 ng/ml)
MCP-1 (20 ng/ml)
MCP-1 (50 ng/ml)
MCP-1 (100 ng/ml)

FIG. 14

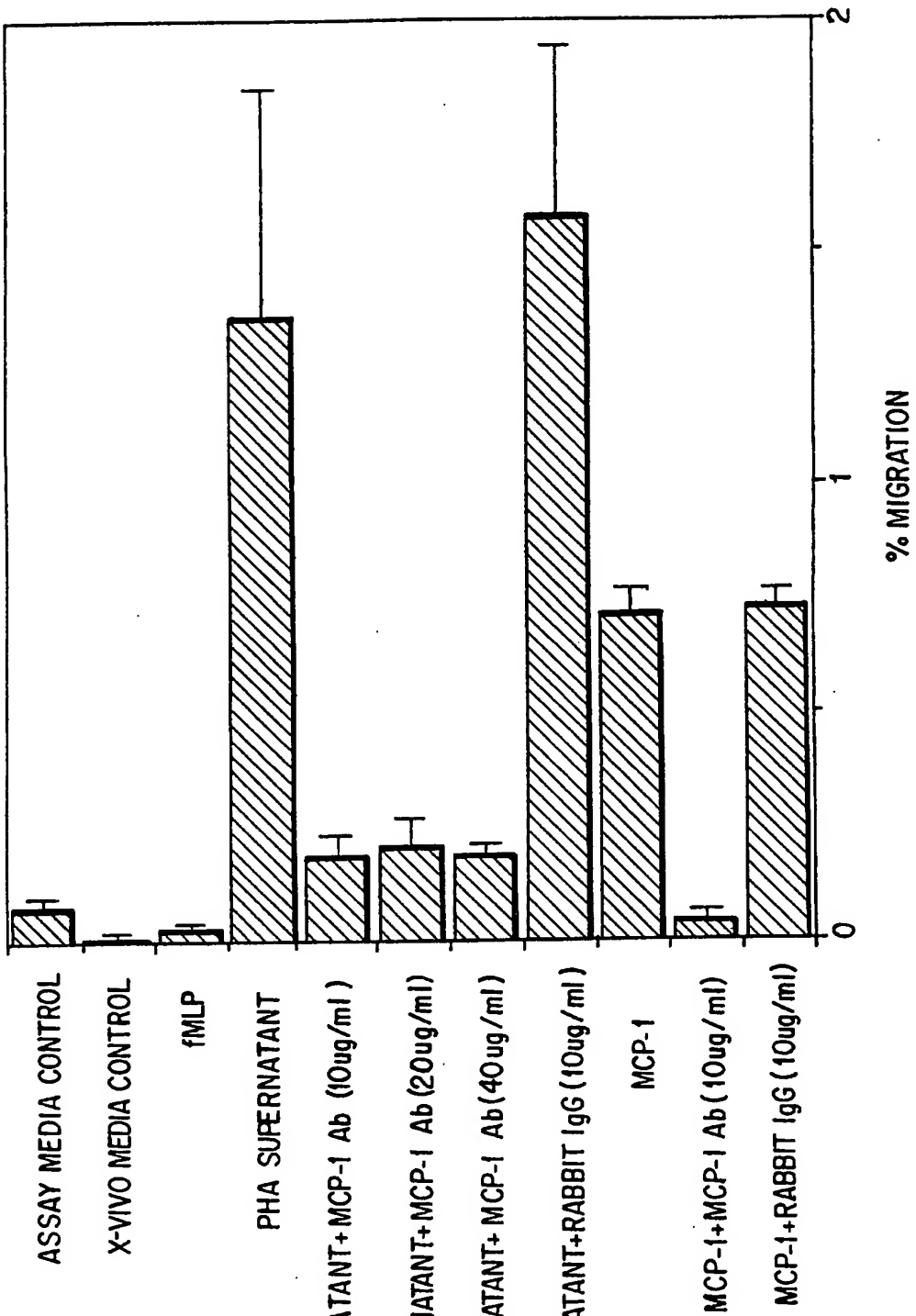
ASSAY MEDIA CONTROL
X-VIVO MEDIA CONTROL
fMLP
PHA SUPERNATANT
PHA SUPERNATANT+ MCP-1 Ab (10ug/ml)
PHA SUPERNATANT+ MCP-1 Ab (20ug/ml)
PHA SUPERNATANT+ MCP-1 Ab (40ug/ml)
PHA SUPERNATANT+RABBIT IgG (10ug/ml)
MCP-1
MCP-1+MCP-1 Ab (10ug/ml)
MCP-1+RABBIT IgG (10ug/ml)
0
1
2
% MIGRATION

FIG. 15

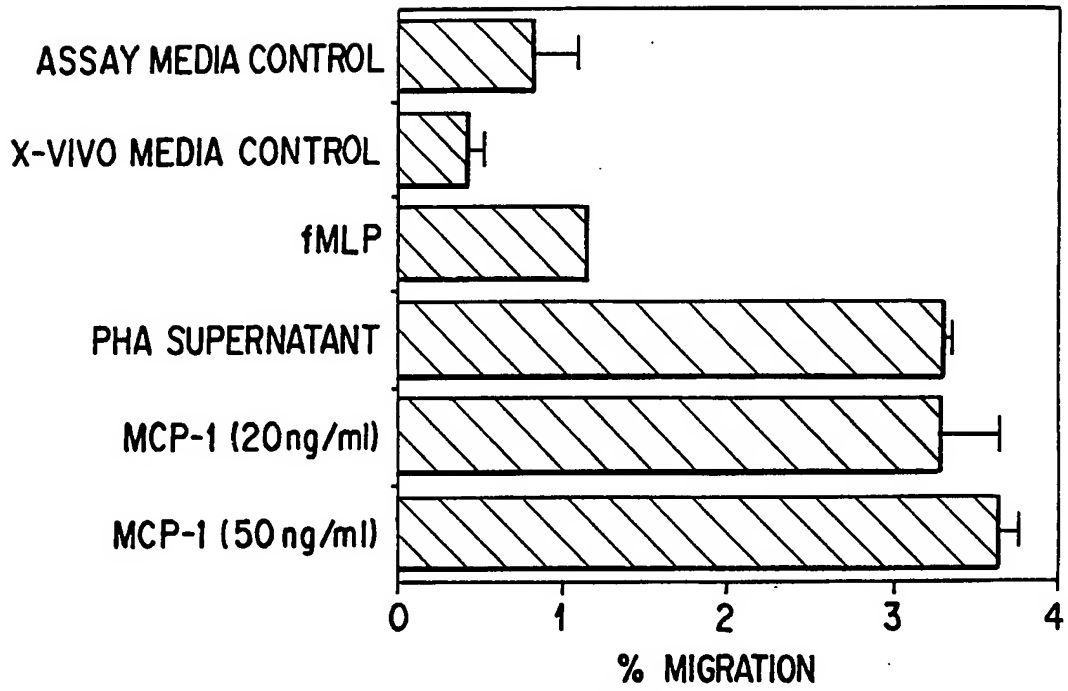

FIG. 16A

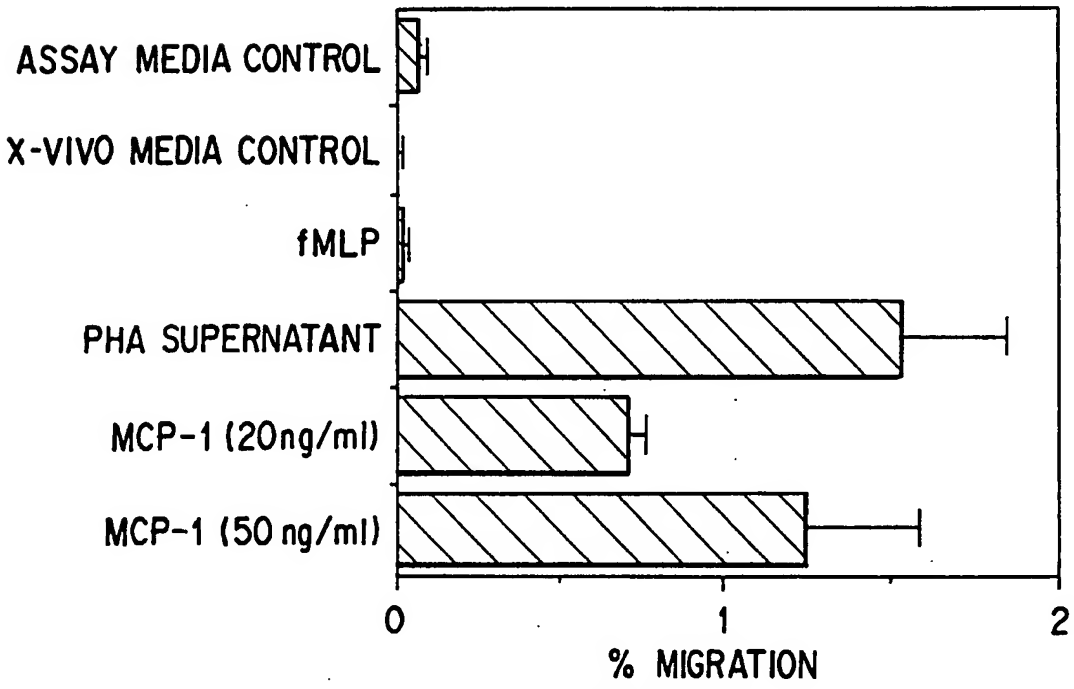

 FIG. 16B

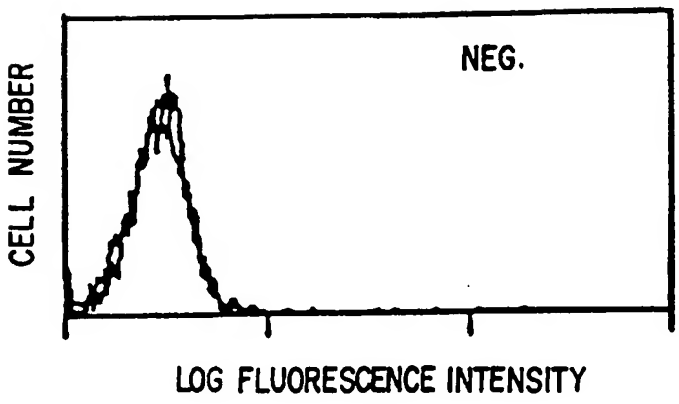

FIG.17A

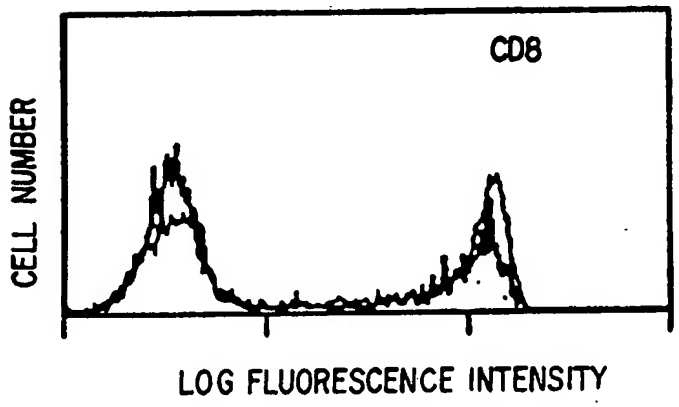

FIG.17B

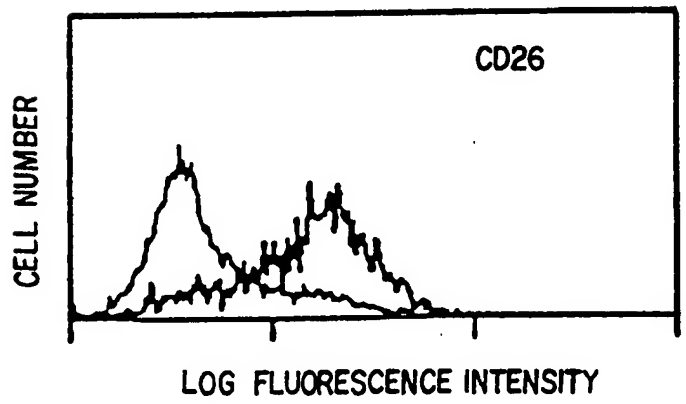

FIG.17C

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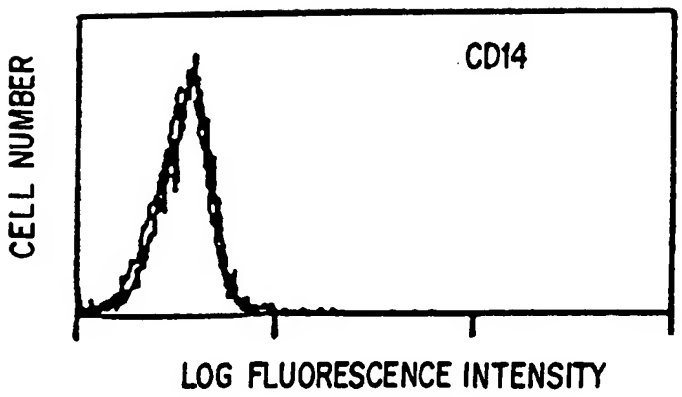


FIG.17D

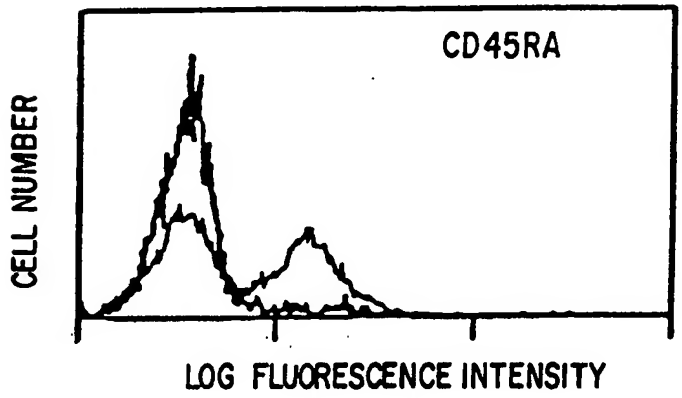


FIG.17E

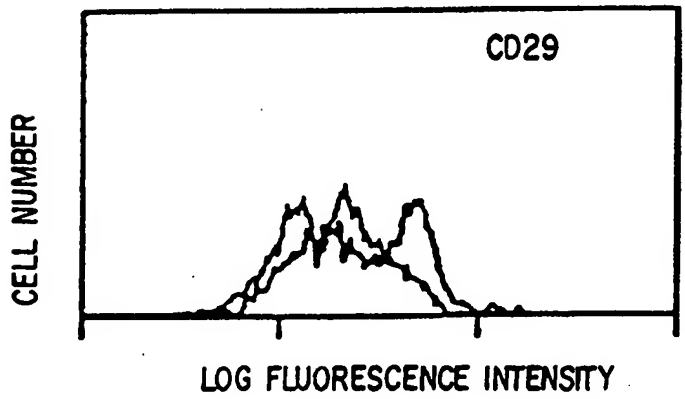


FIG.17F

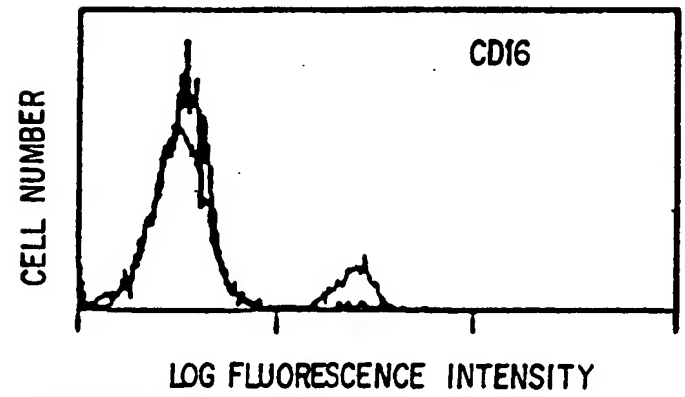


FIG.17G

FIG.17H

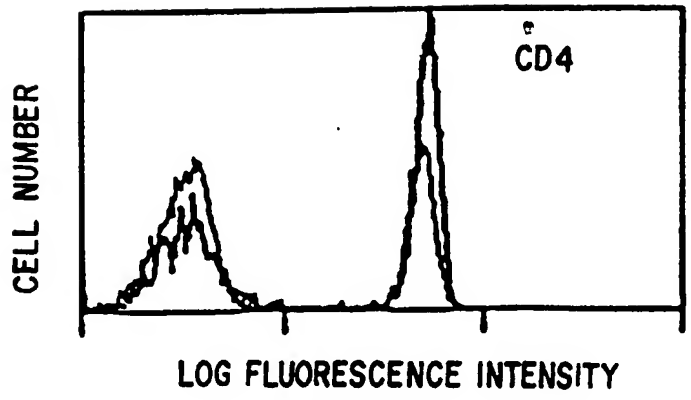


FIG.17I

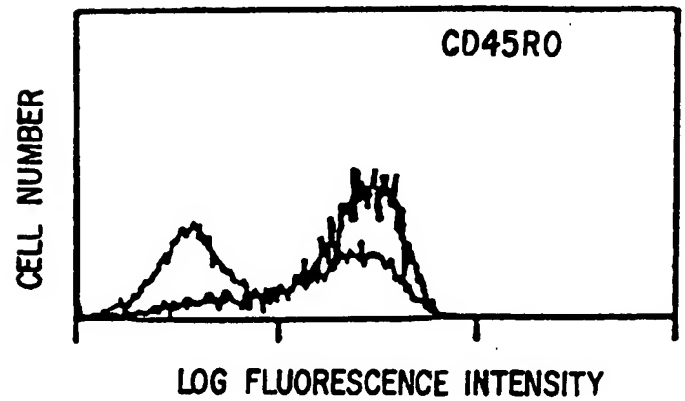


FIG.17J

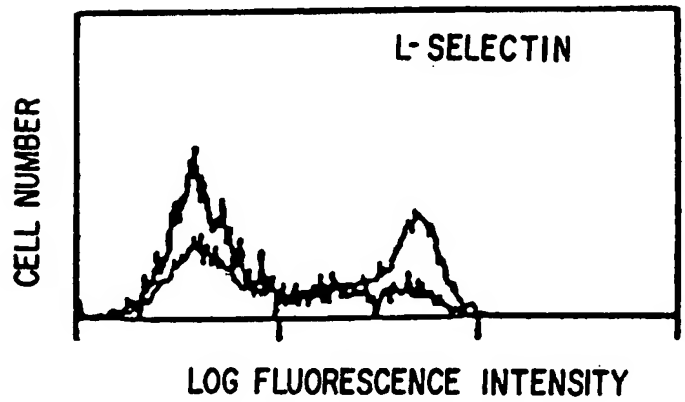
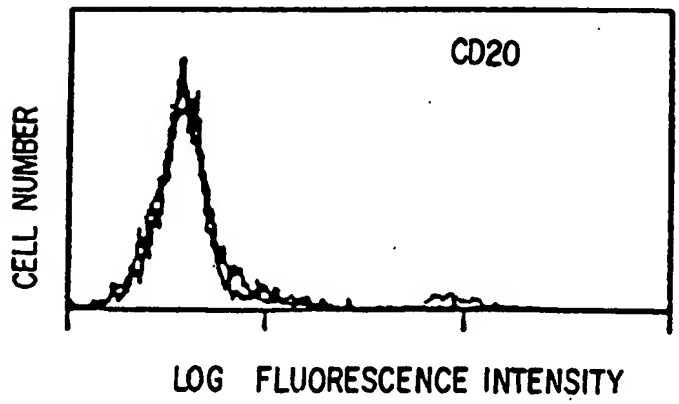


FIG.17K



```
Gln Pro Asp Ala Ile Asn Ala Pro Val Thr Cys Cys Tyr Asn Phe Thr
 1               5                  10                  15

Asn Arg Lys Ile Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile Thr
            20                  25                  30

Ser Ser Lys Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Ile Val Ala
        35                  40                  45

Lys Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val Gln Asp Ser Met
    50                  55                  60

Asp His Leu Asp Lys Gln Thr Gln Thr Pro Lys Thr
65                  70                  75
```

FIG.18

INTERNATIONAL SEARCH REPORT

In tional application No.
PCT/US94/02632

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.24, 29, 240.241, 240.242, 284, 285; 436/63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS: Lymphocyte#(2A)(chemoatt? or chemota?)
BIOSIS: Lymphocyte#(3A)(chemota? or chemoattr?) and endothel?(5A)(monolayer? or confluen?)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Immunology, vol. 146, no. 12, issued 15 June 1991, A.F. Kavanaugh et al, "Role of CD11/CD18 in adhesion and transendothelial migration of T-cells: analysis utilizing CD18-deficient T-cell clones", pages 4149-4156. See page 4150, col. 1, 4th full para.	35-40
X A	Cellular Immunology, vol. 105, no. 1, issued 1987, J.W. Potter et al, "Separation and purification of lymphocyte chemotactic factor (LCF) and interleukin 2 produced by human peripheral blood mononuclear cells", pages 9-22. See abstract; page 12, 2nd full para.; page 17, 1st full para.; and para. spanning pages 18-19.	26-27 1-20,25,41-42

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 APRIL 1994	Date of mailing of the international search report 12 MAY 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer David Saunders Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

Int tional application No.
PCT/US94/02632

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 21-24 and 28-34
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

no computer readable format for the sequence was provided.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

In .tional application No.
PCT/US94/02632

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 39/395; C07K 15/14, 15/28; C12M 3/00, 3/04; C12N 5/06; C12Q 1/02; G01N 33/53

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.8; 435/7.24, 29, 240.241, 240.242, 284, 285; 436/63; 530/388.23, 389.2